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**CHEMICAL
RESEARCH,
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CRDEC-SP-88025

**THIRD ANNUAL CONFERENCE
ON RECEPTOR-BASED BIOSENSORS**

Edited by

**James J. Valdes, Ph.D.
Darrel E. Menking
Mia Paterno**

RESEARCH DIRECTORATE

July 1988

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**U.S. ARMY
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Aberdeen Proving Ground, Maryland 21010-5423

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SECURITY CLASSIFICATION OF THIS PAGE

ADA198966

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION / AVAILABILITY OF REPORT		
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE			Approved for public release; distribution is unlimited.		
4. PERFORMING ORGANIZATION REPORT NUMBER(S) CRDEC-SP-88025			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION CRDEC		6b. OFFICE SYMBOL (if applicable) SMCCR-RSB		7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423			7b. ADDRESS (City, State, and ZIP Code)		
8a. NAME OF FUNDING / SPONSORING ORGANIZATION CRDEC		8b. OFFICE SYMBOL (if applicable) SMCCR-RSB		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423			10. SOURCE OF FUNDING NUMBERS		
PROGRAM ELEMENT NO.		PROJECT NO.		TASK NO.	
				WORK UNIT ACCESSION NO.	
11. TITLE (Include Security Classification) Third Annual Conference on Receptor-Based Biosensors					
12. PERSONAL AUTHOR(S) Valdes, James J., Ph.D., Menking, Darrel E., and Paterno, Mia					
13a. TYPE OF REPORT Special publication		13b. TIME COVERED FROM 87 Sep to 87 Sep		14. DATE OF REPORT (Year, Month, Day) 1988 July	
15. PAGE COUNT 96					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP			
23			Receptor Hybridomas Signal processing		
15	06	03	Toxins Microsensors Signal transduction		
			Biosensors Receptor immobilization		
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Advances in biotechnologies of fermentation and genetic engineering have created the potential for the large-scale production of toxins, creating a new chemical and biological (CB) warfare threat of incredible diversity that existing reconnaissance, detection, and identification methods cannot meet. CB agents owe their extreme toxicity to selective interaction with biological recognition sites known generically as receptors, which are vital for cellular function. A novel, target-oriented strategy has evolved to exploit the fact that receptors are, by nature, extremely efficient detectors of CB agents. The issues are: could receptors, coupled with electronic microsensors, detect all known and unknown CB agents, and, if so, how many receptors would be required? The focus of this meeting is on a systems engineering approach to biosensor development. Specific topics discussed include production of receptors using molecular biological and hybridoma techniques, receptor immobilization and interaction with artificial supports, diverse microsensor technologies, and signal transduction and processing. (RW)					
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED		
22a. NAME OF RESPONSIBLE INDIVIDUAL SANDRA J. JOHNSON			22b. TELEPHONE (Include Area Code) (301) 671-2914		22c. OFFICE SYMBOL SMCCR-SPS-T

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PREFACE

This conference was sponsored by the U.S. Army Chemical, Research, Development and Engineering Center, Aberdeen Proving Ground, MD 21010-5423. This conference started and ended in September 1987.

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THIRD ANNUAL CONFERENCE ON RECEPTOR-BASED BIOSENSORS

Valdes: Welcome to the third annual conference on Receptor-Based Biosensors. When we held our first conference two years ago, it was primarily a contractor's meeting with about thirty people present; it has since expanded considerably. We have been gaining visibility in this program over the past two years and the contractors and people who have been involved in this biosensor work have made some extraordinary strides. This year we're fortunate to have in attendance Brigadier General Peter Hidalgo, the commanding general of CRDEC, the sponsor of the conference. He's going to start the conference with a few opening comments.

Hidalgo: Good morning, ladies and gentlemen, distinguished guests. I would like to welcome all our foreign visitors who have traveled so far to be here and help us in this important program. I must tell you that I feel somewhat out of place. I am not a scientist; in fact, in looking at the program agenda, I couldn't even pronounce most of the titles of the various sessions. Nonetheless, I appreciate the importance of this session and I am excited about the prospects of receptor site technology and the promise that it holds for the Army in the future. The next thing I want to tell you may not be too well received. Research, at least in the Army, at the Chemical Research and Development Center, is not done just for the sake of research. It's done with a purpose in mind, an ultimate objective, and that is to provide our armed forces and those of our allies with enhanced equipment and better defense against the threat of chemical and biological warfare. We have to keep that in mind. Although I know that many of you do research in an academic environment or perhaps in an industrial environment, and that is your total focus, we have to keep in mind that our focus is the American soldier, sailor, airman, and indeed those of our allies who need the products of this research. As you may know, CRDEC is the U.S. Department of Defense lead laboratory for chemical research and development. In response to Army, joint service, and Department of Defense requirements, we conduct all phases of research, development, and engineering for chemical and biological defense. The production and use of a variety of chemical and biological agents throughout the world had made the threat to U.S. forces an urgent reality, perhaps more so now than ever. The use of blister and nerve agents in the Iran/Iraq conflict in the Middle East demonstrates to us vividly that the United States could face chemical weapons in wars other than the war in Central Europe on which we all seem to focus so heavily. There have been reports of use of chemical agents of biological origin by the Soviets in Afghanistan. In keeping with our U.S. national policy we do no work that is related to the offensive use of biological agents in any way, shape or form. The United States had an offensive biological weapons capability in the 1960's, and in 1970 President Nixon declared that we would unilaterally disarm and we did that with dispatch and completeness as verified by the Department of Health and Human Services and many other outside

agencies. Nonetheless, even though we have no biological weapons work, biotechnology had eliminated the distinction between the traditional chemical agents and biological organisms. New agents including toxins, physiologically active compounds, and genetically manipulated organisms are within the grasp of our potential adversaries, even those with modest technological resources. The threat of the employment of biological warfare agents constitutes a significant and permanent change in the nature of armed conflict. We, along with many other nations, subscribed to a convention a few years ago eliminating biological warfare, but it addresses a small spectrum of the biological situation as we see it today.

Prior to the inception of this receptor site program, the Army's strategy for battlefield detection and identification of chemical agents relied on equipment which responded to a known, specific, identified threat. With the advent of recombinant DNA and other advances in biotechnology, the soldier could face a potential threat of incredible diversity: a threat for agents of biological origin. Toxins which are currently exotic neurobiological tools may soon be produced in large quantities using advanced fermentation methods and engineered to have precise psychochemical and pharmacological properties which would make them useful to an adversary for military purposes. Clearly then a new detection strategy was required; a strategy which exploited the fact that toxins owe their extreme toxicity to their selective interaction with physiological recognition sites known generically as receptors. It was this fact that led to the development of a novel target-oriented strategy in which the exquisite recognition capability of receptors, coupled with the transduction and amplification properties of microelectronic sensors, would be combined to produce the first demonstrations of receptor-based biosensors by Drs. Eldefrawi, Chambers, Valdes, and Newman in February of this year. The program received its initial impetus when an advisory panel organized by Dr. James J. Valdes of CRDEC and chaired by Dr. Elias Michaelis met to discuss the merits of this approach. I'm pleased to see that Dr. Michaelis has agreed to chair the receptor session. His brilliant research has been partially supported by the Army Research Office and it is the participation of such distinguished scientists from universities and from industry which will ensure the continued success of this program.

The panel that I mentioned a moment ago answered two questions. First, is a target oriented detection strategy using receptors even feasible? Second, if it is feasible, how many receptors would be required to detect a wide spectrum of potential agents? The answers were encouraging. The military requirements can be met with about twelve receptors. The receptor-based biosensor had been conceived. The program is directed by Dr. Valdes at CRDEC and is organized around several centers. First, the Applied Physics Laboratory here at Johns Hopkins, provides systems engineering and expertise in the area of surface chemistry and materials. Scientists at Johns Hopkins and the Universities of Maryland, Nevada, and Texas at San Antonio, work on receptor biochemistry. Several other contractors including Biotronics Systems and ORD, Inc. provide expertise with various microsensor test beds. As the program continues, we expect to include other researchers including those in the international community. The next such symposium will be held in France.

Gathered in this room are scientists and engineers who are world leaders in their fields. The technological and scientific issues which you will be discussing are not trivial. In fact, they define the very cutting edge of emerging biotechnologies. We need them in the Army and in the Armed Forces to provide us with the protection and ability to detect these exotic new agents. You may know that in the Army today we have no capability to detect biological organisms, toxins, or anything other than the traditional nerve, blood, and blister type agents. For the Army, the success of this program is a high priority because of its potential to save the lives of our soldiers on future battlefields and hopefully to make such battlefields less likely, perhaps even obsolete, by providing a stronger defensive capability which deters the use of biochemical warfare. There are probably as many non-military applications as there are creative minds in this audience, including, for example, industrial process control, medical diagnostics and environmental monitoring. Certainly, that last category is one of great concern and importance to all of us in and out of the military. You certainly have an interesting set of sessions. I wish you the greatest success. At this point I would like to express my thanks to you for having me here this morning and I'll turn the conference back over to Dr. Valdes.

Valdes: I'm going to cover briefly a general introduction to our program and then turn it over to our first session chairman. The real problem we face is that in the next decade, advances in biotechnologies will enable the mass production of the toxins that exist right now to become interesting neurobiological tools. The advisory panel was the genesis for the whole program. The advantage of the receptor approach is that it's target oriented, making it unnecessary to be concerned with particular agents in the environment. If it's physiologically relevant, it's going to interact with receptors. I use the term receptor broadly, to include the classical types, like the acetylcholine receptor, as well as ion channels and certain enzymes and antibodies; anything that is a recognition site. We've carried out a paper and pencil exercise at CRDEC in which we looked at various lists of toxins and chemicals which concern us, cross-referenced all these lists, and then listed with what receptors they interact. As we can see, if we can isolate, purify, clone, express, immobilize, and couple to an electronic device about five of these sites, we should be able to detect about 80 percent of the potential threat agents.

The logical sequence of this work is fairly straightforward. You have to identify which receptors you need; we've done that. You have to produce them; that's being done in laboratories around the world. You have to immobilize and stabilize them. These are large proteins and are not particularly stable molecules. They're very fragile, and have to be in a compatible environment. Once you've done that, you have to couple them to a microsensor. Very few of the electronics engineers know the language of biochemistry, and very few of the biochemists really know anything about the hardware. The first session today is life sciences, and I've asked the speakers to keep in mind that there are engineers in the audience. In the engineering session, which is Saturday, I've asked the speakers to keep in mind the sensitivities of the biochemists. Once the receptors have been coupled to a microsensor, a signal must be generated. The beauty of the

receptor approach is that receptors transmit information. Whether it's tagged optically or translocating ions, the signal must be reduced to the language of the microsensor. The microsensor must amplify that signal and analyze it, and then give a report.

That breaks down into several R&D issues. The first issue is the recognition sites themselves; they have to be produced and stabilized. The next logical issue is the interface. That's where the program may be weak, as few scientists are working at the interface between the biology and the electronics. The third major issue is the microsensor. There are numerous microsensors, including fiber optic waveguides, capacitive sensors, chemical field effect transistors, and optical field effect transistors, to name a few. The last issue is an engineering problem primarily, and that's to package this into a system that will be rugged. The ideal sensor is sensitive. It's specific in that it will respond to what it's designed to respond to and not give false positives. It is reliable; if there is something out there, it should report it. It can be manufactured, and it's small. There are sensors with which we've worked that meet all these criteria. This photograph taken by Dr. Eldefrawi is of an acetylcholine receptor that has been immobilized in a lipid bilayer coated on top of a silicon chip. These are fragile lipid membranes; ultimately we have to put them in some sort of a thin film polymer, but it shows that it is possible to couple receptors and microsensors. We have achieved preliminary evidence that these things will respond to the toxins that normally come in contact with them. On that note, I'll turn the conference over to the first chairman, Dr. Michaelis.

Michaelis: Thank you, Dr. Valdes and General Hidalgo. I would like to take just a moment to introduce the members of the panel and indicate to you that there will be a change in the presentation sequence. Dr. Eldefrawi will present his speech on acetylcholine receptor-based biosensor immediately following my remarks, and I will then follow with my own presentation. To introduce to you the members of the panel sitting at the table: Dr. George Hess, from Cornell University; Dr. Eldefrawi, from the University of Maryland; Dr. Matt Mertes, from the University of Kansas; Dr. John Leonard, from the California Institute of Technology; and Dr. Ramachandran, from Genentech. You have already received a full introduction to the receptor-based biosensor program from Dr. Valdes' perspective, who was the driving force and who conceived many of the principles for making progress in this area. I will not belabor the point; I only want to mention that without Dr. Valdes' efforts, this conference, and much of the work that's going on in this area, would probably not be taking place. It would be taking place in terms of the isolation of receptors and other related studies, but not quite as focused as what we have come to experience in the last few years. An excellent example of the application of basic scientific work to the area of receptor-based biosensors is exemplified by the work that Dr. Eldefrawi will be presenting.

Eldefrawi: General Hidalgo, Dr. Valdes, Dr. Michaelis, ladies and gentlemen. Allow me to start with a few historical notes. The term receptor was introduced about the turn of the century to describe the cellular component responsible for drug action. The principles of chemical transmission at neural synapses were established during the

twenties, thirties and early forties and acetylcholine was recognized as the first neurotransmitter, with many neurotransmitters to follow. In the fifties and sixties, many neurotransmitters were identified, and it became obvious that these chemicals operated through receptors whose function is to trigger cellular responses when they bound their transmitter chemicals. Up to that point, receptors were only a pharmacological notion, a term; there was no biochemical basis or understanding of what those receptors were. In the seventies and eighties, many of those receptors were identified, isolated, subtypes recognized, their molecular properties elucidated, their genes harnessed, and today, receptors are being modified genetically.

This ushers in a new era in receptor research which is to make use of this accumulated wealth of knowledge about receptors to make a commercial product, what I call a biosensor; something that, as Dr. Valdes elegantly explained, couples the high recognition capability of those selective regulatory proteins with microsensors to do the recognition. These would have tremendous implications for a variety of fields, such as medical diagnostics and environmental monitoring. What is the central function of receptors? In our bodies, receptors do indeed function as biosensors. They sense specific chemicals in minute concentrations in a sea full of chemicals, and only when they recognize those specific chemicals do they act as transducers and trigger a response in the cell. Receptors are thus endowed with very sophisticated recognition capability. Consider that one receptor molecule can detect one molecule of its chemical and induce a response, as we can demonstrate with patch clamp techniques. The question is, can we use these recognition capabilities, coupled with the microsensors, to have a working model that can have commercial application? The second part of my talk will convince you that this is not only possible but we do have an extremely good start. Let me go through some of the information for the engineers among us to bring them to the point where we can make some sense of the results of the second part of the talk.

Acetylcholine receptors are the prototypes we use, and these are in the brain, in the skeletal muscles, in various parts of our autonomic organs like smooth muscles, and in ganglia. At least two classes exist: the nicotinic acetylcholine receptor, identified by very fast responses, and muscarinic acetylcholine receptor, that shows fairly slow but tonic responses. Besides the pharmacological separation between the two, there is physiological separation. The receptor we are going to work with and develop a biosensor with is the nicotinic acetylcholine receptor. The two systems utilize two different ways of transduction to tell the cell that the chemical substance acetylcholine is in the environment. The nicotinic receptor operates an ion channel that is a component of the receptor molecule, and when it binds acetylcholine it opens the channel within microseconds to milliseconds. The muscarinic cholinergic receptor operates with a second messenger, where binding of the chemical substance then enhances binding of a nucleotide binding protein; this will then activate a catalytic site, and it is the released second messenger that produces the cellular response. These two modes of transduction are not limited to cholinergic receptors, but include glutamate and GABA, that operate a similar system, and adrenergic, opiate, peptides, hormones and so forth that operate through the second

messenger system. The acetylcholine receptor with which we work comes from the electric organ of fish. It is almost identical to that found in our own skeletal muscle. There are several Torpedo species that are being used by different laboratories. The properties of the receptors are the same in all of them. Purification of the receptor was established years ago. This is the protocol that we used and published in '73, taking the electric organ from the fish, grinding it, and then extracting it with detergent and taking that detergent extract, incubating it with an affinity gel that will then pull out the receptor from the solution and remove all extraneous proteins. Eventually, one can have the receptor attached to affinity beads, and from this one can elute it with a drug like carbamylcholine in high concentration, subject it to a dialysis process, and end up with a pure receptor protein; it takes about 24 hours.

Properties of this receptor protein have expanded; this is one of the old works that both Professor Heuss and I collaborated on when I was at Cornell back in '75, showing the four units of the receptor protein with only the alpha subunit carrying a specific label for the acetylcholine recognition site. This is a purified receptor protein negatively stained to show that even when protein is taken out of its membrane and deposited in a specific protein film on a glass slide and negatively stained, you can see a donut shaped structure like two triangles sitting on top of each other, forming a multisubunit structure. These proteins sit properly in a bilayer. The various subunits, two alpha subunits and a beta, a gamma and a delta, combine to form the structure within the bilayer and extend about 50 angstroms outside the cell membrane, and about 15 angstroms inside. I just came from a meeting in Israel on cholinergic receptors where tremendous details of molecular properties of receptors are being revealed. My message from that meeting was that since we know a great deal about how to harvest large quantities of receptor protein, what are we going to do with it? When are we going to benefit from this information?

When we started two years ago, Dr. Valdes and I talked a great deal about the possibility of using receptor responses to detect organophosphate anticholinesterases. The acetylcholine receptor exists in a variety of conformations, or states, and within the cell membrane there are a minimum of three states: when it is not being activated at all and the channel is closed, when it is activated through binding of acetylcholine, and the system changes its conformation and then a channel is opened that allows ions to flow in along the electrochemical gradient and that produces the response in the cell. A drug like phencyclidine (PCP) binds to the receptor when it is in the activated state and inhibits the action of this receptor. It also stabilizes the receptor into a desensitized state, in which acetylcholine dissociates the receptor, then goes back to a resting state and is ready to receive another message. We can make sealed vesicles that are quite rich in acetylcholine receptor and acetylcholinesterase. They are highly enriched acetylcholine receptor vesicles that would have almost 50 or 60 percent of the total protein of acetylcholine receptors. We could take actual receptor, pure protein, and put it back into phospholipid liposomes and they would look essentially the same. We use a variety of these membrane preparations. The one that I'm going to describe is one that is enriched in both esterase and receptors. We use radioactive PCP as a reporter probe that binds on allosteric sites on

the receptor. In the absence of any activation, i.e., zero acetylcholine concentration, the binding rate and the kinetics of binding of PCP are extremely slow. But as we titrate the environment of the receptor with acetylcholine, rising from 1 nanomolar to 300 nanomolar, you can see the quick change in the kinetics of binding that becomes explosive when one has a high enough concentration to saturate the receptor sites. That increase is due mainly to the increase in rate of association of PCP, the reporter molecule, that goes above two to three orders of magnitude. If one takes the initial rate of this assay at 30 second exposure and plots the response as a function of the dose of the activator of the receptor, a dose-response function that mimics a cellular response to acetylcholine results. This is enough proof to show that the binding of this particular reporter molecule expresses the receptor response. If we look at the effect of carbamylcholine on PCP binding, we find that the two curves coincide and are parallel to the activation of receptor measured by an uptake of sodium 22. We measure the uptake and inclusion of this sodium 22 within the vesicles and the two curves are parallel. Why it is shifted to the right is another point we can discuss later. We take a preparation from an electric organ and, measuring the electric potential across the cell membrane, the active membrane shows that it has a polarized state of -80 millivolts. Adding as much as 2-1/2 micromolar acetylcholine on the cell is ineffective, because the cell has a very high titre of cholinesterase which digests the acetylcholine so fast that the cell produces no response. Once the esterase in the synapse or in the cell was inhibited by an organophosphate, the cell immediately was depolarized by acetylcholine to -10 millivolts. Acetylcholine by itself does not produce much of a response. Inhibition of the esterase by the anticholinesterase is required to make the cell respond very strongly. When a motor nerve is stimulated it releases massive quantities of acetylcholine, only a fraction of which reaches the receptor; the rest is lost to hydrolysis by the esterase. Now we bring in DFP, a prototype of anticholinesterase nerve agent, and put it in the assay at different concentrations, and the curve shifts to the left as we increase the concentration of DFP. By the time we reach 10 micromolar, or even 3 micromolar of DFP, the system has gone from less than 5 percent response to 100 percent response. Practically no response for this low concentration of acetylcholine exists at micromolar ACh concentrations, but there is a response if the esterase is inhibited by an anticholinesterase.

This kind of result was duplicated by Dr. Valdes at his lab at Edgewood. All the nerve agents that we looked at produced this result consistently, and one can then use the receptor response to detect DFP, organophosphates, and other anticholinesterases. Arnold Newman will describe a type of sensor, a capacitive sensor, that will monitor and detect any capacitance change on its surface. They have used the sensor by coupling to its surface T-2 toxin, and then they have attached T-2 toxin antibodies. Proteins have low dielectric constants, and when toxin is added the antibody is removed by competition, with resulting capacitance increases. There was a measurable signal, proving that the principle was viable. We can use capacitance sensors to monitor interaction of a receptor with its ligands. However, as a pharmacologist, I do not see a receptor working out of its environment, because the bilayer is the site of excitability

even in a synapse. These sensors were looked at under an electron microscope and the interdigitation between the fingers of the sensor can be seen. On the surface of the sensor we build the bilayer by first putting a monolayer of lipid and then interfacing that with another monolayer of lipid that carries the receptor molecule. We hope that the receptor molecule will interface well enough that, when it is activated by acetylcholine, a conformational change will take place and perturbation of the bilayer will occur. This would allow water molecules and ions to get closer to the surface and the capacitance sensor would detect a change in the response to receptor activation. We used a capacitance bridge from APL and Dr. Valdes' laboratory, and an electronic lift controller so that we can produce the exact speed that we need to give the best possible interface. A tensiometer is used to determine the amount of liposomes to be placed on the second monolayer. There are two types of liposomes that we put on the sensor. The particles in the membrane taken from either one interface or the other are quite different from the smooth surface of liposomes that do not have protein.

To give you an idea about the properties of the capacitance sensors we have used at least three types indicated here. This indicates only a change in the surface film; in other words the main construction of the sensors is the same, but only the surface film is different. You can look at the capacitance measured in air, in the absence of any kind of hydroelectric constant material except what the sensor might pick up from the atmosphere such as humidity. These are very low in picofarads but once you start putting it in even a very low hydroelectric constant media like n-hexane, it increases slightly, but when put into something like water or water containing phospholipids, it jumps very high. A phosphate-buffered saline either alone or containing a high concentration of acetylcholine will do the same. You can see that the different surface chemistry is very important in that any given surface would produce a response quite different from the others. Type 3 was the best and that's the one that we use to collect data on interfacing receptor protein with the capacitance microsensor. This was an elaboration of the very first experiment that Drs. Mansour, Valdes, Annau and I did in February. This got an extremely strong response to acetylcholine but no response whatsoever to d-tubocurarine, a drug known to block receptor response to acetylcholine. The antagonist produced no response in the system, whereas the activator or the endogenous neurotransmitter produced a response, and a dose-response system at that. We consider this a successful point. We've changed the interface and now get even higher sensitivity and better results. We measure membrane response to buffers or buffers containing different concentrations of acetylcholine and find they are extremely stable over long periods of time. However, if we have a biosensor with a receptor in it, we see a slight difference in the biosensor with the protein and this is anticipated because the protein in the bilayer creates some perturbations. But it is the response to acetylcholine that is dose-dependent in nature, and that is expressive of receptor function, recognizing acetylcholine at one micromolar. The observed fading phenomena is essentially what a cellular response gives; a strong response which fades slightly because of receptor desensitization. Remember those three states. The receptor, after

activation, desensitizes and that starts blocking entry of any more hydroelectric material that contains the high capacitance.

We took a preparation from the electric organ, which is a tissue that has only cholinergic receptors, and tested it against six other neurotransmitters. It responds only to acetylcholine. All these neurotransmitters that operate in the mammalian brain and in many other preparations do not see the receptor, and the biosensor does not respond to them. This is only the liposomes' interface. We have bilayers but no receptor protein. Compare that with the response to the various transmitter substances, and in most cases there is a negative value to acetylcholine. What would happen if we take a tissue that is multisynaptic, that has other receptors and interface it? Dr. Sherby has taken a synaptic preparation from rat brain and made synaptosomes. These are nerve endings which carry most of the receptors extracted from the detergent cholate. Rat brain's response in the buffer is very similar to the Torpedo biosensor with only acetylcholine receptor, but all of the receptors respond, indicating that this very crude preparation works. We've made biosensors for GABA, glutamate, norepinephrine, dopamine, and 5HT, but we should take each one individually and show selectivity for its ligands. It does bring into focus one thing: that on a single biosensor we can place a dozen different receptor systems and each one of those would respond to a group of drugs that has its own pharmacology. A single sensor can have a very large range of detection capability. I would like to impress upon you that this biosensor on this flat sheet operates or responds almost like a cell. Now, if we take the preparation with the biosensor treated with Naja alpha-neurotoxin which is known as an irreversible blocker of the acetylcholine receptor, and then measure capacitance in response to acetylcholine, nothing happens. In the free system, response is extremely fast; it stabilizes and is constant for about 30 minutes. If we expose the biosensor to d-tubocurarine, a slowly reversible blocker of the acetylcholine receptor, we get very little response. But while the sensor is sitting in the exposure bath and the system is perfused with acetylcholine, we slowly see a return of the response as DTC dissociates from the receptor. Acetylcholine activates the receptor and we see a capacitance increase. This is almost mimicking a cell's or a muscle's response to acetylcholine.

Can we detect nerve agents or opiates, using this kind of a sensor? Yes. A comparison of a response of a biosensor that is acetylcholine-based to acetylcholine jumps from 0 to almost 4 nanofarads. A very strong signal stabilizes within 5 minutes.

And if we use a preparation that comes from an enriched brain fraction that has esterase, and then inhibit the esterase with DFP, we get a much heightened response, almost double the signal that we get with a fairly low concentration of one micromolar acetylcholine. We can, even with fairly primitive instrumentation, interface a receptor protein with a microsensor. Now, I'm not claiming that in a patch clamp you can detect one nanomolar. But I believe that as we develop a better system of interfacing, we can push the sensitivity two or three orders of magnitude. It has the potential to apply to a variety of receptor systems and it has a potential of detection not just of receptor drugs but other drugs as well.

I would reiterate that I think these kinds of results usher in the new era in receptor research. And before I leave you I would

like to acknowledge those who have contributed and worked with us in the last two years. I start with my colleague from Johns Hopkins University; Dr. Zoltan Annau. I already mentioned Dr. James Valdes, with whom some of the crucial experiments were done. My first graduate student and now professor in the Department of Chemistry at the University of Alexandria, Dr. Nabil Mansour, was instrumental in getting that first successful experiment done. Dr. Sherby, who is currently a post doctorate fellow working with me, provided most of these later results. And then our colleagues, the engineers, Mr. Arnold Newman, currently of Biotronics Systems Corporation, who was the one to convince me that capacitance sensors have a chance, and Drs. Blum and Andrea of the Johns Hopkins University, who are currently working with us on the second generation of biosensors.

Q: How long can you keep using these particular biosensors?

Eldefrawi: Right now, about a day. Once you put them in solution they are stable for hours.

Q: And the sensitivity you've gotten is about 10 micromolar?

Dr. Eldefrawi: No, 1 micromolar.

Q: So you're in the parts per million region.

Eldefrawi: We have a long way to go yet.

Q: I'm Dick Taylor, from Arthur D. Little. We filed a patent application earlier this year on a new, thin film membrane technology for immobilization of receptors onto capacitors. One of the examples that we've given is acetylcholine. We have a thin film that stabilizes a receptor at a minimum of 6 months. We have been able to get response to cholinergic ligands within 2 to 3 seconds on it. So this is something I think might be of interest to this audience.

Eldefrawi: We get responses within the second. It does take some time to stabilize, and I'm quite sure as we improve or change the surface chemistry that we're working with, we're going to see not only faster response time, not only longer stability, but higher sensitivity.

Q: If I understand your talk correctly, you seem to believe that these sensors are most sensitive under conditions where the normal ligand for the receptor is present and you're looking for inhibition of that rather than direct interaction with some unknown with the receptor. Is that correct?

Eldefrawi: In a way, yes.

Q: If that's true, given the receptors normally desensitize in time, how are you going to know when to introduce the natural ligand in order to detect an unknown?

Eldefrawi: Well, with this system you have to realize that once the receptor has been activated, it is the change in dielectric constant of

the surface of the sensor that's detected. If the receptor desensitizes after that, the sensor will continue to detect that high capacitance. At least at this stage we are not washing the system back to a baseline again. So I am not worried whether the receptor desensitizes or not. I worry if the receptor is activated or not. Now the second part of your question, how you detect an agent that would be present. I'll put it in the same solution with the activator. If it is present there it will interfere. It will either reduce the signal or increase the signal. All we're saying is, the pharmacology looks good, the chemistry is working.

Chambers: Dr. Eldefrawi, do you have in your on-going experiments an idea as to how many receptors you're putting down on the surface and the response that you're getting? How does that translate in terms of active receptors versus receptors that are not optimally oriented?

Eldefrawi: We're putting in several billions of receptor molecules, at least, and the fraction that is responding may be less than one percent. We have not yet gone to that quantitative aspect of the surface chemistry to correlate the size of the signal that we see with the number of receptors.

Q: How constant does the chemical environment have to be and what changes in buffer concentration are tolerable before it obscures the signal?

Eldefrawi: We don't know yet. You notice that the one buffer that we have used all along is the phosphate-buffered saline. It's a high dielectric constant media.

Q: May I suggest one of the ways you could look at the amount of active receptor you've got on your sensor? You can get radiolabelled antibodies to the alpha subunit, and find out how many molecules are on the surface. Then you could use your PCP assay to find out how many of those receptors are functional, giving you a quantitative angle on the function's surface.

Eldefrawi: We are doing this already in a different way, but that particular way would reveal a lot of information as well. And I hope that this kind of presentation will trigger interest in several areas, such as modification of the alpha subunit or even working with a piece of receptor protein rather than the whole complex.

Q: Do you have any firm ideas as to what the correlation is between receptor and ligand interaction and its capacitance change at the molecule level? What's really causing the capacitance change?

Eldefrawi: It will probably be a couple of years before the physicists and the engineers come up with a firm answer to your question. I believe that the perturbation that occurs in the receptor protein in association with the bilayer would allow not just water molecules but ions to reach there. What we're looking at and calling a capacitance measurement is probably a capacitance plus conductance. I am not certain, but I'll let my colleague, Dr. Andreou, answer that.

Andreou: There are multiple effects that can cause a capacitance change, the most prominent of which is the dielectric constant region. This local region on the surface will correspond to capacitance change. Initially, some of the questions were that there were side walls that chemicals bind on and we found through several experiments that's not the case. It's more of a surface effect. That's what happens when you introduce other materials to the solution. It sounds as if the binding event causes some gross change in the hydrophilicity of either the receptor or the surrounding lipid membrane. That, in turn, changes ionic content. In the new system we have designed, we will have a curve to measure all parts of the changes, not just the capacitance, but conductance.

Eldefrawi: Allow me to add to what you said Dr. Andreou, since he already mentioned about what happened to the bilayer. Remember, when you put the bilayer in a solution like a buffer phosphate saline, it comes to equilibrium very quickly; not just with the water in the media, but with the ions in the media and some of those ions will saturate sites not only on the outer layer but also the inner. In the control biosensor, the capacitance increases somewhere between 16 and 20 nanofarads and stabilizes there. That stabilization is the equilibrium point of the forces and the response to receptor activation is the result of this perturbation that allows redistribution of these charges and water. Now, the environment on the surface of the sensor has changed because of receptor activation. We don't know how much of this is sensed as capacitance coming through the receptor channel. The important thing is that we get the response, and the time frame is pharmacologically correct.

Q: In one of your drawings you show the electrodes being covered with an insulating material. Is that the way this sensor is set up? That should be a very small capacitor, so you're looking at a small capacitor in series with a very large capacitor, which is your lipid bilayer.

Eldefrawi: That's right.

Q: It should be extremely difficult to see changes in the capacitance because of the very large capacitance of the bilayer.

Eldefrawi: There are three types of capacitance sensors that we've looked at. There was such an increase in capacitance with water or phosphate-buffered saline that you can't see anything. Once you put the reconstituted bilayer on another type of sensor, you put it in the aqueous media, it will increase without any activation of the receptor. A combination of factors is allowing us to see these signals, and you cannot argue with success. I have a system that is pharmacologically successful. It recognizes the drugs the way a cell that carries an acetylcholine receptor does. Dr. Amira Eldefrawi wants to make a comment.

Eldefrawi, A.: The receptor in the lipid can be stored for months and maybe years.

Q: How frequently do you have failures in reproducibility?

Eldefrawi: In the last batch of sensors that we have used, response to a basal condition without receptor or response to reconstituted receptor base system has been within ten percent error, between one sensor and the other. The sensors and the system of surface chemistry used are quite stable and giving us this reproducible type of response. If you put a microelectrode in muscle cells, as much as 10 percent variability in the membrane potential from a microelectrode appears. Now, they are fabricated using thin film technology.

Michaelis: I would like for a moment to reminisce about the beginnings of this whole endeavor of looking at receptors and ion channels as possible biomacromolecules that could be introduced in sensor development. The very first time that that notion was presented to me was by Dr. Valdes when he had come to visit me at the University of Kansas. It was in the middle of winter and at that point we had been snowed in for several days. I thought maybe he was dreaming, but I'd never thought that the idea was out of place or it didn't have substantial merit. It took the effort of many scientists coming together to develop some guidelines about the steps in the development of such biosensors. And what I would like to do today is trace with you some of that history. Some of these points have been discussed in detail by Dr. Eldefrawi and Dr. Valdes. I will start with why the receptor ion channels might provide appropriate means of detection of toxic chemicals or biological toxins. The selectivity for the ligand binding sites of this receptor has already been mentioned by Dr. Eldefrawi as being very high, and it also has a very high affinity. So it isn't only selectivity but also high affinity, usually in the submicromolar, nanomolar, or even picomolar range, which is an advantageous aspect of using receptors for biodetection. The stability of the ligand recognition site was mentioned by Dr. Eldefrawi. Tissues can be frozen for a considerable period of time, and the ligand recognition capacity can still be detected in those tissues. One aspect of the receptor is the high turnover number, especially for ion channels. The receptor class which has an ion channel component may have as many as a hundred thousand to one million ions going through that channel upon activation, and that can become a signal. The problem with that type of strong signal is that there may be unstable components of a receptor response. The final reason is that many receptors are targets for toxins and toxic agents, and because of this they represent a good starting point for biosensor development.

The acetylcholine receptor site is probably one of the most frequently targeted areas for neurotoxins. Some examples are given: histrionicotoxin, which is acting at the channel of the acetylcholine receptor, d-tubocurarine, which Dr. Eldefrawi has already mentioned, and of course the various snake toxins, as well as the organophosphorous compounds which act post-synaptically at the acetylcholinesterase site, but may also have some interaction at the receptor site. So, receptors are targets for multiple chemical as well as biological toxin activity. Another receptor system or ion channel system is the voltage-dependent sodium channel. Saxitoxin and tetrodotoxin interact at the opening of this channel. Batrachotoxin,

scorpion toxins and, more recently the various snail toxins also interact with the voltage sensitive sodium channel. A last example of a receptor channel component that is a target is the chloride channel associated with a GABA receptor complex. That one is susceptible to inhibition by picrotoxinin, bicuculline, and other drug agents and chemical derivatives. So here are three examples of frequently targeted sites that can be used in the construction of receptor-based or ion channel-based detectors. This is a partial list and it somewhat matches the list that Dr. Valdes presented to you of the receptor sites that were targeted by the panel of scientists that examined all of these issues. It lists the acetylcholine, GABA, catecholamine, indolamine, and peptide receptors, and a variety of different channels such as the sodium channel, which is an example of a toxin-sensitive or blocked site.

If receptors are a good micromolecular species to incorporate in a sensor device, what are some of the issues that one is confronted with? The first is that you need to incorporate either multiple receptors and ion channels or have devices that contain at least one of the various classes that were indicated. Large numbers of these proteins are also needed. Dr. Eldefrawi showed you how easily one can go, in terms of purification, from a homogenate of a tissue to the introduction of the receptor into a biosensor. Any large-scale production would soon deplete most of our Torpedo in the Mediterranean region and possibly some other species. The techniques of molecular biology will be important in developing methods for larger production of these various proteins. Selection of an appropriate signal, such as ligand binding or ion channel gating event is of concern. For example, in the device that Dr. Eldefrawi mentioned, it may be a combination of both of these events that are being detected, or maybe one type of event versus the other. And, finally, the stability of the macromolecules is important with regard to whether ion channels can be easily maintained, and under what conditions of storage.

I would like to review the advances that have been made recently which give us increasing hope that many of these aspects of making receptor-based detection devices may be met. The procedures for the isolation and reconstitution are remarkably similar for a variety of receptors and ion channels. Slight modifications, different ligands, different properties may work better, but the methodology seems to be very well developed and working quite well for a variety of proteins. Tremendous strides have been made in this approach. The contributions of molecular biology have allowed not only the characterization of the primary structure of these proteins, but also the expression of these proteins in other cells. Dr. Hess and Dr. Leonard are going to present their work on the voltage-sensitive calcium channel. Another issue is the chemical similarities between receptors and ion channels. Nature has some strategies for making families of these macromolecules. In the presentation that Dr. Ramachandran will give, you will see examples of nature's approach to this. The sodium, calcium and other voltage-sensitive channels are likely to reveal properties which are fairly common among the various categories. These properties are also in natural receptors, synthetic receptors, or synthetic ion channels. Some of the work that Dr. Mertes will be presenting to you will deal with attempts to mimic enzymatic and other types of macromolecular interactions. Finally, another issue

is similarities in the chemical properties and the types of actions and the sites of actions of the toxins, because they may reveal to us commonalities of the target and the site of the various toxins. That might give clues for synthesis or design of toxin sensitive sites.

The classic picture that has emerged over the past few years has been that of the cholinergic receptor. Dr. Stroud's laboratory has shown this type of structure and how they are arranged in a lipid membrane surface. You can see them as small doughnut-shaped macromolecules in an enlarged structure and the concept of the nicotinic acetylcholine receptor as a pentameric structure. It is inserted into the membrane and contains within it the ion channel component activated by acetylcholine, another agonist. I would like to focus on some of the similarities that are emerging between this prototype receptor and some of the other receptor families which have ion channels associated with them. An example is Dr. Barnard's paper on the GABA receptor which appeared in Nature just a few weeks ago. Their model is what that receptor probably looks like in the membrane. It is a tetramer made out of two alpha and two beta subunits. It and the ACh receptor have some uncanny preservation of similar regions, even though these two receptors recognize two different types of classes of agonists. They have a different type of ion channel; this is a chloride channel as compared to the sodium or potassium channel of the acetylcholine receptor. The beta loop similarities in the structure of the transmembrane domains of this receptor with about a 50 percent homology and a high degree of preservation of similar structure, indication that there are some common themes in both the ion channel component and the external domain which may trigger the opening of the ion channel or recognize the ligand. Beta loops and ion channel characteristics have been revealed for the glycine or strychnine receptor, a 48 kilodalton protein. The strychnine receptor has been isolated and the DNA clone has been sequenced. These three receptors which have been studied in detail represent one type of family.

Another sort of family would be receptors that use the second messenger system about which Dr. Eldefrawi spoke. The mechanism of muscarinic interaction works by receptor activating the G protein system, and the G protein system then activates a potassium channel. A similar type of system exists with rhodopsin, utilizing G protein system to activate a phosphodiesterase, and cyclic GMP being the controlling influence in the channels. The prediction would be that the muscarinic cholinergic receptor, and other receptors such as the adrenergic receptors which use the same second messenger system, would have similarities in structure. The rhodopsin and beta adrenergic receptors reveal a high degree of homology in the seven transmembrane domains for the beta adrenergic receptor from human brain tissue. This second family contains the recognition area and has interaction with G proteins. The voltage-dependent calcium channel is about 140 to 170K molecular weight, versus the voltage-dependent sodium channel at 180K molecular weight. Nature has conserved a substantial degree of information in these macromolecules; the primary structure of the voltage-dependent calcium channel and the sodium channel have similar transmembrane domain arrangements. This makes a large molecule that forms the ion channel, retaining one helical structure in each one of these four transmembrane domains; one helical structure which is

positively charged, represented by multiple arginine residues in that structure. This appears in the calcium and voltage-dependent sodium channels and seems to be the site where voltage sensitivity is sensed. Dr. Catterall's proposal shows that these positive charges in this helical structure up out of the plane of the membrane allow the opening of the channel.

There is still a need to identify, isolate, and purify more receptors. We have tried to identify a glutamate receptor from brain tissue to show that a protein complex we have been isolating and studying has a high glutamate binding capacity and a high affinity for glutamate in the submicromolar range. The glutamate binding protein has a molecular weight of approximately 70K for the major band and 63K for the second band. We are using this protein as a possible model for a brain glutamate receptor, and are seeing if it falls in the aforementioned class of receptor/ion channel systems. This synapse from brain tissue shows the side of transmitter release for the synaptic vesicles, and the post-synaptic side; and using antibodies raised against these 70K dalton protein we get labelling at the synaptic membrane region. Also, when this protein is reconstituted into liposomes as Dr. Eldefrawi described, activation by glutamate causes a sodium influx into the vesicles. This response disappears if one pretreats the vesicles with detergents, and is only seen when the protein is incorporated; liposomes without the protein, or other proteins such as albumin, do not give this glutamate-activated sodium flux response. We have reconstituted this protein into a system that can give us a quasiphysiological response using a liposome system and patch clamp technique, and we have been able to get signals activated by glutamate. These signals are usually of a duration of around 1 millisecond, and with higher concentrations of glutamate we get a greater variation in both open times of the channel and frequency of appearance. This is the type of protein that we are currently trying to use to develop probes for cloning purposes. We're probing DNA libraries to see what type of structure we will eventually be able to reveal for this particular protein and whether it falls into that family of receptor ion channel systems.

The last part of my presentation is to draw attention to the toxins, the site of the toxins, and what this is telling us about these macromolecules and the sites of interactions. We know, for example, that saxitoxin and the tetrodotoxin interact with the voltage-gated sodium channel. An important component of molecular structure is thought to interact with a carboxyl group at the opening or at the top of the channel. This seems to be prevalent not just for the saxitoxin and tetrodotoxin molecules, but also for other types of toxins. Adenine blocks a calcium-activated potassium channel and these two residues, 13 and 14, are very crucial. Arginine residues have one group of arginine and if these 2 residues are replaced with glycine, that destroys the activities. The epsilon amino group of lysine cannot quite operate in the same fashion as the arginine residues in this particular toxin. A further demonstration is from a paper by Dr. Usherwood; these spider toxins that have been recently isolated are identified as blockers of glutamate receptor-activated ion channels. Sodium channels and the glutamate receptor have some commonalities in arginine, which is the terminal residue in at least two of these toxins, a type of polyamine structure being present there. The amino

acid residues in the glutamate receptor ion channel systems are interacting with these toxins in some way.

An idea of receptor-based and ion channel-based biosensors a few years ago seemed a bit far-fetched. A great deal of progress has been made and I hope that the presentations you will hear today will give further impetus for some of these ideas in development of biosensors.

Eldefrawi: How easily would you think it would be for the group of receptors that operate through second messengers to interface with that type of sensor compared to the ion channel type receptors? Do you anticipate the second messenger type receptor would cause more difficulty?

Michaelis: I think they might be easier because if you could interface it with the activation of an enzyme you could have a photochemical type of reaction, which you can detect fairly easily. But this puts an additional burden on trying to have not only receptor protein, but having the transducing proteins plus that enzyme system. That will create an additional problem. Since progress has been made in terms of the G proteins in these systems and in the isolation of some of the enzymes, it's certainly feasible. It will require a great effort and you could possibly link the DNA message for all of these proteins into one long DNA molecule and transcribe the whole array together. It's a theme one should explore rather than trying to isolate and introduce three distinct systems.

Q: The glutamate receptor is available in only very small quantities. Would you comment on the difficulties of making ion flux measurements or some of the single channel current measurements which you mentioned in your presentation. What is involved; what does one have to do to make these measurements?

Michaelis: The success in finding one of those ion channels to measure the response is low. In our measurements we estimate that our success currently is somewhere between 4 percent, 5 percent at best. The success ratio is considerably better when making many vesicles. I would say that, from the first step of purification, we get a success ratio that's greater than 80 percent of the time. It does consume all of the protein that you can purify for one single experiment. The accumulation of data is very difficult because of relatively low yields of protein and a signal that is not that strong.

Q: Do you know which type of glutamate receptor you've isolated?

Michaelis: From drug specificity, it looks as if it is the glutamate quisqualate type receptor. We have interaction of glutamate, aspartate, quisqualate- the best of the agonists. MDA is weaker; you have to go to high micromolar, almost half millimolar concentration to get near 50 percent of ligand binding.

Q: Have you tested something like APV or APB?

Michaelis: Yes, those have very, very low activity, about 20 percent or so inhibition at high concentrations.

Q: Arginine is particularly talented for forming ion pairing type complexes with carboxylates, even phosphates. It seems that some of the free energies for ligand binding with arginine versus a substance like glycine are more than an order of magnitude.

Michaelis: Right, stronger. There may be a key carboxylate group in the opening on the external face of the ion channel where these toxins are binding. This may be a common site for a variety of toxins. We're going to continue this morning's presentations with the presentation on "Chemical Kinetic Measurements Using Fast Reaction Techniques in the Investigation of Neuronal Receptors", and Dr. Hess will present the paper.

Hess: Dr. Valdes, Dr. Michaelis, ladies and gentlemen. I never heard about biosensors before this morning and I've had some time to think about how the techniques that we've developed can be useful. In general, protein is of interest to the use of fast reaction techniques because proteins undergo fast changes in state with altered ligand binding properties. What made it possible to investigate reactions in solutions was the development of fast reaction techniques. We have been interested in developing similar reaction techniques that can be used on cell surfaces. We're also using the acetylcholine receptor as a model for developing these techniques. The reaction between cells in the nervous system consists of the binding of the ligand, which can be acetylcholine or a whole series of other neurotransmitters, to the receptor, forming a complex. Then this complex undergoes a conformational change to an open channel form allowing ions to go through the membrane. It's responsible for the electrical signal and the transmission process, and this is characterized by an equilibrium constant K . In addition to this conformational change, receptors usually undergo another conformational change which can occur in as rapidly as 10 milliseconds. This is called desensitization, and this inactive receptor form has altered ligand binding properties. The reason it is important to make measurements rapidly before this conformational change is that the information is based on its inactive form. In all types of slow measurements, one would get constants characteristic of the inactive receptors. It is this field where fast reaction techniques are important. To give you an example of its importance in the development of biosensors, the substances that you developed as regular toxins bind to a strongly inactive receptor form. You would need acetylcholine to convert the receptor to this form, so when the receptor is in its natural state, these substances would essentially escape detection. What is important are these dissociation constants and the parameters that control the whole thing. Only a fraction of open channels determine whether a signal is transmitted or not. There are many clinical and commercial compounds and toxins that modify this reaction. We would like to get information about real constants that determine how they affect the channel opening process. Similar constants are modified in many diseases affecting the nervous system. This is a reason for using fast reaction techniques

and developing techniques capable of making measurements of these constants in the millisecond or microsecond time range.

The first technique we developed mainly to identify cells containing specific receptors; there's an optical technique, for instance, measuring the activity of receptors in cells. We are interested in the fly receptors because it is genetically well characterized and many diseases show abnormal receptor functions in the fly. How does one detect a cell containing a specific receptor so that one can study this particular cell? You've heard Dr. Michaelis mention the difficulty of making studies with a glutamate receptor because it occurs in small quantities, but these techniques allow you to make measurements directly with the cell containing such receptors. Finally, I will mention our new expression system for producing receptor proteins in large quantities, enough for structure determination and physical characterization, and also isolation of receptors for other purposes. You all know about the oocyte expression system which requires microinjection of messenger RNA into each oocyte. The idea in this system is that once the cells are transformed into yeast cells, they can be grown in large quantities and keep on producing receptor proteins.

Mammalian type muscle cells contain acetylcholine receptors. These cells can be made fluorescent by equilibration with a fluorescent dye, which we synthesized. The fluorescence of the dye is quenched by cesium ions. These cells are in the medium in which the sodium has been replaced by cesium ions. As long as the receptor channels are not open, the cells keep on fluorescing. When we add carbamylcholine, the fluorescence quenches in these cells. Certain cell types have been identified in the cell culture by sticking electrodes into each cell and then adding chemicals in succession. With this technique we have a cell culture containing either acetylcholine receptors or glutamate receptors. We can detect if this particular cell contains either glutamate or acetylcholine. The optical techniques can be used further. We can use several reagents sequentially; the fluorescent signal is maintained for at least 3 to 4 minutes. Since the fluorescent quenching also depends on the rate with which ions flow into the cell, the technique can be used to get an estimate of the number of receptors on the cell surfaces. If the flow of ions into the cell is modulated by toxins or clinically interesting compounds or modulators, we can detect this as well. The rate with which a fluorescence is quenched is the rate with which the cesium ions move into the cells. The next thing is to determine these constants in the general reaction leading to the open channel form. It takes only two constants to account for the concentration dependence of acetylcholine or carbamylcholine over large concentration ranges. We have cornered a concentration region of 10 thousand and it is similarly possible to get all kinds of information about the action of inhibitors on the native non-desensitive form. We could tell whether the two inhibitors bind to the same side or whether they're inhibited by binding only to the open channel form of the receptor. The technique has been used to study chemical modifications of the receptor; for instance, phosphorylation of the receptors is very important. With natural modification we can determine what factors phosphorylation has on receptor function. Eldefrawi has used these fast reaction techniques to study the effect

of lipids on these constants and how they effect the opening of channels. Let me show you the old techniques which we developed first.

Acetylcholine receptors exist in very large quantities in solution, and it is possible to isolate the protein and incorporate it into membrane vesicles. Once we have the receptive membrane vesicle, one can measure the acetylcholine-induced influx of ions into membrane vesicles. This is done by using mixing devices in which the membrane vesicle and the acetylcholine inhibitors are mixed in the 5 millisecond time region. We could show that, by appropriate uses of mixtures and fluorates, it is possible to do this without breaking membrane vesicles. With a quench flow technique, acetylcholine is allowed to flow into the membrane vesicle for a 5 millisecond time period and then the reaction is quenched. It is possible to determine the number of ions that have flowed into the membrane vesicle mixture as a function of time. In another technique, we use acid which has been allowed to equilibrate inside of the membrane vesicles and quenching is due to cesium ions. Neither one of these methods is really possible with most of the other receptors unless we isolate the receptors, incorporating them into the membrane vesicles.

We developed a technique with cells so that one can make chemical kinetic measurements with a high time resolution and not have to isolate receptors by themselves. A cell impaled by an electrode and the whole cell current recording techniques have been described in detail. What we do is to flow a solution over the cell surface. In general, rapid perfusion techniques have been used to measure rapid desensitization of the receptors in cells. Rapid desensitization was discovered using rapid chemical reaction techniques with membrane vesicles of electric eel. What is different in our technique is that we've corrected for the desensitization process, to correctly observe current for desensitization that occurs as the cell equilibrates with ligand coming through the solution flowing over the cell. Rate-limiting in flow is the rate with which a layer flows over the cell surface. The observed current is usually measured at 5 milliseconds. In an experiment in which carbamylcholine is flowing over the cell surface, we obtain the flat line where desensitization has been corrected from the observed current. What we get from the current amplitude is the same information one gets from ion flux measurements. We can determine the fraction of the channels in open channel form as a function of ligand concentration. From this, we can calculate the parameters important for determining the channel opening. Also, we can determine the effect of modifying reagents and clinically important compounds and toxins on receptor function. Chemical modifications of the receptor are gotten from this current amplitude. All the problems can be solved if measurements are made as a mixture of receptors in various states as the various ligand binding constants and properties. I think that this technique should use at least four different cell types containing all kinds of different receptors so the measurements are not restricted to the acetylcholine receptor. These other receptors, like glutamate, GABA and glycine, can be used without having to isolate the protein and incorporating it into lipid vesicles. The time resolution of the technique I discussed is only about 10 or 20 milliseconds.

The question is: how can we get information about the elementary steps in a reaction? The rate constants pertaining to the

ligand binding steps and channel opening and closing are addressed all in one experiment, and we can get information about how these constants and parameters are modified by compounds that modulate the reaction, or by diseases. A cell is impaled by a whole cell current recording electrode and we add to the solution an inactive precursor, carbamylcholine. This cell is now bathed in a solution of a completely inactive precursor, and we can photolyze this compound to an active ligand within microseconds. There are three phases of the reaction. For instance, a rising phase of the current gives a low ligand concentration. A rate constant of the ligand binding process at higher concentration gives the rate constant attaining the channel opening. Closing the amplitude of the current gives the same information as you obtain by the flow methods, so we have an independent measurement of the current amplitude, and the falling phase of the current again gives you the information about the rate of desensitization. Ortho-benzyl derivatives have been developed as a protecting group in organic synthesis. These have been used in making inactive precursors of all kinds of phosphates. We have now started to make inactive precursors of neurotransmitters. Instead of using ortho-benzyl derivatives, we use ortho-benzyl phenolglycine derivatives, that is, one of the hydrogens has been replaced by a carboxyl group. This not only makes the reaction faster, but also makes the compounds more soluble. We get a carbamylcholine derivative which is photolyzed at a rate constant of 20 thousand per second, and we have now made derivatives of glycine and GABA. In this case we make an ester derivative of the protecting group and again photolysis rates have turned in half-times of microseconds. In the case of carbamylcholine, we know that the precursor is really inactive and does not interact with the receptor part of photolysis.

We have now the same information with the GABA derivative and all the other derivatives that have all been synthesized recently. I should mention that the first derivative took four years and all the other derivatives were done in the last 3 months. We haven't done the photochemistry except for the GABA and carbamylcholine derivatives. A cell attached to a recording electrode has been equilibrated with a precursor of carbamylcholine; that is, a derivative of carbamylcholine. This is a current recording following a flash with the laser. The current rest time occurs in the 2 millisecond time region of 200 micromolar carbamylcholine. The falling phase of the current gives information about the desensitization reaction. We can resolve the elementary steps in ligand binding and channel opening using this technique. We are now able to not only make measurements with these electric organ acetylcholine receptors, but also with the receptors incorporated into vesicles where the measurements can be made with all types of cells. We have made measurements with brain cells containing acetylcholine receptors, GABA receptors, and glycine receptors. The type of information has to do with elementary steps in channel opening and ligand binding. One can measure the effects of inhibitors or toxins on these reactions. In people who have some sort of abnormal receptor functions, this could be used to determine which of the constants has been affected by a particular disease.

Let me now change the subject and go to another area of research that has to do with the production of receptors in large quantities. The success of using phagocytes has been well documented as demonstrated by the experiments of Barnard and Lester. I assume if

people would like to have biosensors, large quantities of receptors will be required. We decided to use yeast. Once we can transform a yeast cell, we have a stable line that will produce receptors indefinitely. Is it possible to transform yeast cells? The question is, can a completely foreign protein be expressed in yeast; does yeast have some mechanism for inserting such a protein into plasma membrane? And the answer is yes. I'll just give you one example of such an experiment. In this experiment a yeast cell has been transformed. The cell has been treated with specific monoclonal antibody for the receptor and, in order to visualize the monoclonal antibody, a fluorescent antibody is added against the antibody. As you can see, there is a large difference in fluorescence, and fluorescence is indeed due to the specific reaction between the monoclonal antibody specific for the delta subunit. I have done single experiments now with the alpha and gamma subunit and, in fact, we now have a yeast cell that contains all three subunits inserted into one cell. We have a very interesting problem for those who are using yeast as a tool in biotechnology. The beta subunit is expressed in small quantities. So how do we develop beta subunits? What we've done with the alpha subunit of the acetylcholine receptor is to produce it in the same quantities as the receptor is produced in electric organ of eel. We've taken the later sequence and signal sequence of the alpha subunit and put it in front of the structural unit of the beta subunit. These experiments are still in progress and the outcome is unknown, but it will be possible to produce other subunits in large quantities by appropriate combination of chemistry. The yeast can express receptor proteins and insert them into the plasma membrane. We now know that at least a single yeast cell can synthesize alpha, gamma and delta subunits and insert them into the membrane. We also know that the yeast glycosylate receptors have the right amino acid residue. And we also know that the yeast synthesizes the alpha subunit which contains the ligand binding site. Which part of the receptor is important for expression and membrane insertion? This is a problem we are working on and in which the DNA sequence is important for protein assembly. You can see this is a question beyond expressing receptors in yeast. Essentially, one of the major problems in studying receptors has been to determine the constants pertaining to the channel opening process. It's an inactive form which has different ligand binding properties, very often ligand spurning properties, which dominate the measurements. So by making these measurements in the millisecond time region, we can determine its constants, the effect of modifying agents on these constants, and if in any particular disease which these constants could have been affected. Until recently, these sort of measurements have only been possible with receptors that existed in large quantities, such as the acetylcholine receptor. I think some of the techniques I mentioned allow us to make these same measurements with all types of receptors; at least all neuroreceptors that are activated by amino acids. There are about ten or twenty types of receptors, of which little is known.

Q: I have two questions. The first is biological. Why yeast rather than an animal cell?

Hess: Well, techniques for growing yeast in ton quantities are known. I don't think you can grow animal cells in ton quantities.

Q: My other question was on the cell flow technique. Is the cell dropped through liquid?

Hess: No, the cell is suspended by an electrode at a hundred-eighty degrees from the direction of flow. And the other principle is a series of flow over spherical objects; we just used that theory to make the appropriate corrections for the rate of flow of the liquid, the rate of transfer; and the material from the liquid to the cell surface to correct the observed current of the desensitization process that occurs during equilibration.

Q: Dr. Hess, with regard to your yeast work, the acetylcholine receptor is a glycoconjugate. It has an antiglycoside complex; yeast do not synthesize complex oligo-saccharides but they do synthesize oligo-saccharides. Obviously, the system works. Do you have any information as to how the differences in the glycosylation affect any of the properties of the receptor in the yeast?

Hess: Well, so far, we have only measured the effect of toxin binding, and the replacement of toxin as ligands on the yeast cell. There don't seem to be any differences that we can detect by these techniques. We are not yet at the stage where we really assemble a functional protein. We have no idea about the different glycosylation even though the right side is glycosylated. This won't make any difference in the final assembly of protein. People at MIT are now looking at what the glycosylation process is in yeast. I think it has been shown that, for the acetylcholine receptors, the glycosylation event is not absolutely a natural requirement for biologic activities. Is that correct?

Eldefrawi: Well, I'm not certain yet as to what has been shown in terms of responses of binding.

Hess: This is a beautiful system because the glycosylation in yeast is such a different architecture than the oligosaccharides found in the mammalian systems. It gives you a beautiful tool to look at folding processes of membranes and insertion of membrane bound receptors.

Q: When you've finally solved the problem with the beta subunit, one would just assume then that you have a chemically excitable, yeast line. Do you care to speculate what chemical excitability introduced in an unexcitable cell will do to it? Would it kill it? Will it grow faster? Will it have any affect of future application of fermentation in industry?

Hess: What happens once you add acetylcholine to these cells depends. Some yeast cells are very sensitive to ions and it may well kill it after you open the channels, but other yeast cells are not, so they may tolerate some ion exchange before becoming sick. We use galactose promoters so that the yeast cells are first grown in large quantities then we turn on the genes so they will start synthesizing these foreign proteins.

Q: Think of the columns of electroplax cells from an electric fish as a small biological battery. A couple thousand of those cells are capable of producing seventy-two hundred millivolts of current. In series and in parallel they can generate up to two hundred volts of current. Now, assuming that your yeast have become chemically excitable, each one of them resembles an electroplax, and you have millions of those in series. Would you say that we can make a biological battery out of yeast cultures responding to a chemical that you trigger the battery on?

Hess: Well, interesting idea, I haven't thought about it.

Q: Yeast cells are very rugged cells compared to most cells. It would seem possible to have a whole library of receptors inserted into a very rugged cell like the yeast cell and immobilize the whole cell on the surface, and then use that as a detector.

Hess: Yes, I think one of the easiest ways of combining yeast cells with a fluorescent technique would certainly be very interesting as a biosensor.

Michaelis: We're ready to start the afternoon session. The first speaker will be Dr. John Leonard from the California Institute of Technology and he will be presenting on "Xenopus Oocytes by Exogenous mRNA".

Leonard: Thank you very much, Dr. Michaelis. Ladies and gentlemen, I'm going to be talking about three subjects: calcium channels introduced in Xenopus oocytes by injection messenger RNA from rat brain, sodium channels, and the use of this Xenopus oocyte as an electrophysiological assay system for the cloning of the serotonin 1-C receptor. Norman Davidson and Henry Lester's laboratories at Cal Tech have been collaborating in an effort to study neurotransmitter receptors in ion channels using techniques of molecular biology and electrophysiology. To this end the Xenopus oocyte can serve both as an electrophysiological assay system for identifying ion channels produced by injected messenger RNA, and also for studying structure and function correlations in greater detail. During the middle part of the talk, I'll talk about voltage-dependent tetrodotoxin blockable sodium channel introduced by the same injections of rat brain messenger RNA. The final topic is the use of the oocytes as a cloning procedure where high affinity radio ligands are not available.

Xenopus, the South American frog, is a hardy laboratory animal. It was originally introduced into Southern California in the 40's as a hospital lab test for pregnancy, and stayed on as a pet. The Xenopus oocyte is a large, one millimeter in diameter, frog egg. It has been the system of choice for the expression of messenger RNA after injection. This is mainly because of the large size and prodigious translational capacity. The oocytes are covered by follicle cells and blood vessels. These are removed by collagenase treatment of the oocytes. Then we use a glass needle with a 20 micron tip diameter to introduce 50 to 70 nanogram quantities of messenger RNA in volumes of 50 to 70 nanoliters; in other words, milligrams per mil, into the

oocytes. After two to four days, ion channels appear in the surface membrane. Calcium channels are of particular interest to neurobiologists because they form the primary transduction mechanism between electrical activity of the nervous system and a change in the function of the cell. This occurs because calcium exists at a low level inside the cells, less than 10^{-7} molar, whereas the external bath contains a higher concentration, so an influx of calcium can change concentrations and activate dependent enzymatic reactions inside the cells. On the topic of rat brain RNA-induced calcium channels, I'll first describe how we isolate calcium channel activity from the ion channel activity that is produced by introducing messenger RNA from rat brains into frog eggs. Then I'll describe the calcium channel activity in detail, and talk about the pharmacology of the calcium channels and what we know about neuromodulation by neurotransmitters in second messenger systems. Even though I'm talking about a voltage-dependent ion channel, that's not to say that it's unaffected by any sort of neurotransmitters. On injecting messenger RNA from rat brain, a variety of ion channels are introduced into the membrane. All the studies were done using two electrodes, one to monitor the voltage across the frog egg and the other to pass current to maintain the voltage at the desired level. This produces a voltage step from a holding potential of -80 millivolts to +20 millivolts. The opening of ion channels can be measured as the amount of current that the voltage clamp must supply in order to keep the voltage constant. The voltage pulse is maintained throughout this time period after the step. In order to isolate the calcium channel activity, all of the barium currents through calcium channels were done in the presence of puffer fish TTX toxin to block the voltage-dependent sodium currents. Cesium pretreatment of the cells eliminates much of the contaminating outward potassium currents that would interfere with this measurement. All of this inward barium current through calcium channels is blocked by cadmium, an inorganic agent, leaving only an outward current. They're not really a problem except at more depolarized voltages. When these cadmium-insensitive currents are subtracted from polar currents (under a voltage type experiment) we're left with the cadmium-sensitive barium current through the calcium channel. There's not much difference except at the more depolarized ranges. The peak current which occurs in the membranes depolarized to about +10 or +20 millivolts is about the same whether or not potassium currents are blocked. The calcium channel activity which begins to be activated at about -40 millivolts and peaks at about +10 or +20 is still inward even at +50 millivolts. Potassium currents are seen after these calcium channels are blocked. At a slower sweep speed this current can be seen to undergo a very slow but partial inactivation, with a time constant of about 650 milliseconds. This is one way of identifying this sort of calcium channel compared to other types. The conclusion that we have barium current through voltage-dependent calcium channels is further supported by the high sensitivity to blockage by cadmium. The smooth curve represents a single-sided competitive inhibition curve. The K_d for cadmium is about 6 micromolar. This was not dependent upon the voltage at which we examined the barium through calcium channels. In a similar series of experiments, nickel was found to have about 100 fold higher K_d , and less ability to block than cadmium. This kind of difference

between blockage by nickel and cadmium can help to distinguish different calcium channels subtypes.

In contrast to this high sensitivity to blockage by cadmium, we've tried a variety of organic calcium channel blocking agents including nifedipine, which is a member of the dihydropyridine class, compound W7, which is the only calcium channel blocking agent that works in paramecium, and verapamil. These were completely ineffective. This is the case even when the cells were maintained at a depolarized level to allow the calcium channels to enter a potentially higher affinity state. These particular barium currents through calcium channels were recorded after injecting hippocampal RNA. The hippocampus has a preponderance of dihydropyridine binding sites. The currents we record are identical in the presence or absence of nifedipine.

This is a kind of calcium channel which is nifedipine insensitive. Insensitive calcium channels are often thought to be involved in neurotransmitter leaks. This is one characteristic of presynaptic calcium channels involved in neurotransmitter release. One of the previous talks mentioned omega-conotoxin from the marine snail, Conus geographus, which blocks certain kinds of calcium channels. It is also thought to be involved in neurotransmitter release. This channel is not blocked by omega-conotoxin, so there are several kinds of calcium channels. At this level we're using injections of rat brain messenger RNA, and the current does respond. In a variety of electrically excitable cells, neurotransmitters and second messenger systems have been described that will modulate voltage-dependent calcium channels. We tried increasing cAMP levels inside the cell by pretreating the cells with phosphodiesterase inhibitors and then exposing them to phorbol to increase intracellular cAMP. Although this kind of a treatment affected potassium currents, it had no effect on calcium channel activity. In contrast, when we used phorbol esters which can activate protein kinase C, it caused about a 40% increase in the calcium channel activity. The wave form was not affected and the current-voltage relationship when the current turns on and becomes maximal also was not affected. It's not shifted, it's just enhanced, so the currents are larger in any given membrane potential. Similar sorts of findings for calcium channels in Aplysia cell membranes have been found. One interesting difference is that all the other vertebrate calcium channels that have been studied in peripheral neurons, such as dorsal ganglia, show a decrease in calcium activity and a blockage of the calcium channels by phorbol esters. This is another indication of calcium channel diversity.

Xenopus oocytes are cells and they have some endogenous currents, although quite small. They measure 20 nanoamps compared to about 400 nanoamps for a rat brain RNA induced calcium current. This current has quite different properties as well; it's a transient kind of barium current. The presence of an endogenous calcium channel in a Xenopus oocyte means that one must introduce enough messenger RNA to swamp out the endogenous currents, so the current of interest can be isolated. There aren't any voltage-dependent sodium currents in oocytes, fortunately, although an occasional frog will have a very small TTX blockable current. Different kinds of calcium channel activities can be introduced into the Xenopus oocyte, depending on what messenger RNA is injected. The brain RNA-induced calcium channel shows

a slow 650 millisecond partial inactivation and is quite resistant to depolarizing holding potential, so it's not inactivated very readily, even up to -20 millivolts. The currents peak at about the same membrane potential though. They are all very sensitive to cadmium, especially the brain RNA induced current. Dihydropyridines such as nifedipine are ineffective on this calcium channel. A-kinase enhancement has no effect either, but enhancement of C-kinase by phorbol esters is seen with the brain current. It is possible to get an omega-conotoxin blockable current in oocytes. It's quite clear that there are a variety of different kinds of calcium channels in the nervous system. One of these is a presynaptic calcium channel because it's insensitive and the time course of inactivation of the current is similar to the time course of inactivation of the current seen for calcium 45 uptake in rat brain synaptosomes. In chick dorsal ganglion cells which have been very well studied, there are three different kinds of calcium channels: one that is activated at very low voltages and produces transient currents is called the T type calcium channel; another type that is activated at higher voltages and more depolarized levels is called the L type, for long lasting; and a third is called N, for neither transient nor longlasting. I refer to our channel as NEN because it has similar characteristics to the N type channel, but has about 100 fold slower inactivation time constants, so I call it Not Exactly N, NEN.

There are at least four different kinds of calcium channels, and based on the pharmacology we can find a whole variety of different calcium channels and neurons. So far all I've been talking about are the two electrode voltage clamp studies. It is quite clear that the capacity of charging at time constant 2 milliseconds is very slow compared to some currents of interest; for instance, the voltage-sensitive sodium channel. The way to get around that is to use the patch technology, in which we remove the outer membrane by shocking the cells in hypertonic saline. This produces a gap between the plasma membrane and villa membrane, and then we strip off the villa membrane manually with forceps. We expose the plasma membrane with a large diameter patch pipette that has a 20 micron opening. We can form gigaohm seals to service the cell. We call this the big patch method. It increases the time resolution of recording fast currents almost 10 fold. I'll be recording from only 1-10 thousandth of a surface area of the oocyte so the current scale will change from nanoamps to picoamps. The classic kind of patch technology is the single channel recording. This is done with micron tip diameters or less, both in the cell attached configurations and patches. With outpatches we can see much smaller currents and look at single events. These recordings are from calcium channels recorded at the single channel level at two picoamps, done in an excised outside patch with 70 millimolar barium buffer, and TTX to block sodium channels. Inside the pipet head was a solution similar to the inside of the cell. During a voltage pulse from -80 millivolts to +20 millivolts where calcium channels are activated, the channels will open. On repolarization of the membrane back to -80 millivolts, channels quickly deactivate. The channels are also as likely to be open in the middle of the 60 millisecond pulse as they are at the end. They are very reluctant to undergo an activation. Concerning the voltage-dependent sodium channel, some of the molecular aspects of the way we can change the wave form depend on what parts of

the sodium channel we introduce into the oocyte. Under the standard two electrode voltage clamp that I described, there is a slow capacity time constant, about 2 milliseconds. This isn't good enough to measure sodium currents. The disadvantage of using a very large cell with a huge membrane capacitance is that it needs to be charged. We see an inward sodium current followed by a maintained outward current. This inward current is completely blocked by tetrodotoxin. This is a classic voltage-dependent sodium current.

There are ways to decrease this huge membrane capacitance by using a big patch to isolate a relatively small portion of the membrane. Under a big patch recording, we get better time resolution. These kinds of sodium currents resemble those found in squid axons and other classical studies. We can even see the current reversing, going outward when the membrane is very depolarized beyond the equilibrium potential for sodium. These sodium currents are recorded in normal saline not containing barium. Cadmium was present to block any calcium channel activity. Potassium currents were also blocked pharmacologically. It's producing normal types of channels in the oocyte, activating it by -40 millivolts, peaking at -10 millivolts and reversing, going from an inward to outward current at more depolarized levels than the sodium equilibrium potential. This is known because we know the intracellular sodium concentration. We can produce appropriate sorts of sodium currents in the oocyte, so we decided to attempt a structure-function correlation for the voltage-dependent sodium channel. This was done by size fractionating the messenger RNA from rat brain. A represents the longest mRNA molecules and K the smallest coming off the gradient. T stands for the total unfractionated messenger RNA. When these are run across agarose cells side by side, blotted with nitrocellulose paper and then probed with a radiolabelled probe, we find that the alpha subunit of the sodium channel mRNA exists in fractions D and E, and in total unfractionated messenger RNA. Biochemical reconstitution studies have indicated that the rat brain sodium channels should be composed of one large alpha subunit and two lower molecular weight beta subunits, called beta one and beta two. We know the size of the messenger for the alpha and beta subunit messenger since the beta subunit messengers are so much smaller. We probe these by using another mRNA probe from rat brain in the 2.2 to 2.4 kilobase range. We can see that the messenger RNA which encodes the small beta subunits is found in fractions H and I and not in fractions D and E. We've separated the low molecular weight RNA which encodes the beta subunits from the high molecular weight RNA which encodes the alpha subunit.

Now that we've demonstrated that it's possible to fractionate out the high and the low molecular weight RNA encoding alpha subunits and the smaller beta subunits, we wondered if we could demonstrate any functional role for the lower molecular weight subunits. It is well established that alpha subunit RNA alone could produce functional sodium channels. Although the channels were functional, we wanted to see if there was any role that could be demonstrated for the low molecular weight components. Fast sodium currents come from the unfractionated messenger RNA from rat brain. These are the classic sodium currents we expected. When we injected fractionated high molecular weight RNA which would encode the alpha subunit, but not the smaller beta subunit messenger, the currents inactivate more slowly and

do not reach baseline at all for the high molecular weight RNA. This kind of effect could be an artifact from the fractionation procedures, so we repooled the fractions and added the low molecular weight RNA to the high molecular weight fractions. When that's done, the normal sorts of sodium currents are reconstituted. We can see it's not just a damage artifact. In total unfractionated RNA, sodium channels open at the beginning of the pulse and do not open again during a 12 millisecond pulse. When high molecular weight RNA is used, the channels have a tendency to reopen several times during this pulse. That explains the microscopic currents which are sums of these individual traces of single channel recordings. When the low molecular weight RNA is pooled with the high molecular weight RNA; again, the channels tend to open just at the beginning of the pulse and then do not reopen. This indicates the single channel basis of the slowing of the current. A variety of other characteristics of the sodium current were the same for both the high molecular weight and the total unfractionated RNA-induced sodium channels. They peak at the same voltage, both have the same K_0 for blocking by tetrodotoxin, and both are affected in the same way by scorpion toxin, but the wave form is twice as slow for the high molecular weight fractions. The conclusion we draw is that either we have separated out the beta subunits which would account for the slower decay time, or that some other low molecular weight RNA encoding of a protein that's involved in post-translational processing or modulation of the channel has been separated out.

Finally, I would like to discuss a different topic, which is the cloning of the serotonin 1-C receptor using a system which does not rely on protein purification. The classic kind of a cloning scheme would use protein sequence information to produce information about DNA sequences which could then be used to make all the nuclear type probes to screen a library. The alternative would be to produce antibodies to the protein and then use those to screen library and expression factors. Yet a third possibility exists when used as a functional assay that does not rely on a full length clone. We're able to either produce a messenger by selecting out from a total population with our cDNA clone, or we can actually inhibit the message, depending on which one we select. RNA is first fractionated through gel, so we have different size classes of RNA. The size of RNA which is enriched for serotonin 1-C receptors can be identified by applying serotonin to the oocyte. Then we take just this size RNA, and use it to make a directional cDNA library. First, there are so many that they have to be pooled to about 20 clones per pool. Single strain cDNA's are then hybridized with total mouse brain RNA or serotonin 1-C receptors. The nucleic acids were separated by cesium density gradient centrifugation. The unhybridized DNA, just DNA alone, banded at the top the mRNA, cDNA hybrid bands were in the middle, and the RNA which was not hybridized banded near the bottom. When RNA from this middle band, the RNA-cDNA hybrids, as injected into oocytes, we found a hybrid selection of which the response to serotonin was much enhanced compared to other clones, and when we injected the hybrid depleted RNA there was very little response. That's the case where there is a cDNA clone present in the population to hybridize with the mouse brain. Of course, this indicates that the mRNA for the serotonin 1-C receptor is present in the band in the middle, although it's not completely gone because

there's still some current. When these 20 clones are rescreened and separated out, a single clone called D9 was found which codes for part of the serotonin 1-C receptor. We have a hybrid depletion experiment in which clone D9 was able to arrest the unhybridized mRNA injections to the level of less than 20 percent of the negative clone response, so this signals down to about 20 percent of those seen with any other clones or any kind of controls. The hybrid selection experiment is one in which about a 10 fold higher current was seen when the DNA RNA hybrid band from the middle of the gradient is injected into oocytes. This indicates both the specific selection of the serotonin receptor and its depletion from the total RNA population. The full length clone and sequence are being pursued now. This protein is present in less than one in 50 thousand in rat brain, so there is not the presence of a particular ligand to make a ligand protein complex, especially in the case where there are several similar ion channels that are not identical, but would still bind the same way.

I will summarize the advantages of using the Xenopus oocyte translation system. It's easy to voltage clamp; these are huge oocytes, a millimeter across, and it's quite possible to use these as an electrophysiological assay for cloning. It provides a standard test environment, and any ion channel we want could be introduced into a Xenopus oocyte in the same membrane environment. It can be patch clamped, and this enables us to do both single channel recording and high resolution microscopic current recordings. It is possible to design your own ion channel by either leaving out subunits or doing site directed mutagenesis on the sequence for a clone channel, or exchanging subunits between similar but separate molecules, for example, Torpedo, or calf hybrids with the acetylcholine receptor for the different alpha, beta, gamma and delta subunits. The last procedure that I discussed shows another use of the system, and that is as an electrophysiological functional assay system for cloning where the proteins are not available in an abundant supply and there's no special ligand for isolation and monitoring the cloning process.

Q: Being particularly interested in the binding of saxitoxin and tetrodotoxin to the voltage-activated sodium channel, I understood at one time that removal of one of the beta subunits led to preparations that were no longer responsive to binding.

Leonard: There must be differences between what's required to stabilize the sodium channel alpha and beta subunits and detergent reconstituted systems and what happens functionally in the oocyte.

Q: In your experiment with the sodium channels, an alpha subunit and the betas are removed, and you showed that an activation is removed. Do you also see spontaneous activation of those channels that's reminiscent of what happens with things like batrachotoxin and brevetoxin?

Leonard: There is perhaps a preponderance of a bursting mode when just the alpha subunits are present in the oocyte, so in other words, this kind of bursting mode would be the only one that's seen with just the alpha subunit. But there is a preponderance of non-bursting modes regularly. The analysis is too early to say for sure on that point.

Q: In your calcium data, you're seeing what appears to be one type of calcium channel. In the rat brain itself there will be several types of calcium channels. Why do you think only one is being transcribed?

Leonard: There are some discouraging possibilities: that the oocyte won't process them properly; or that there is just very little RNA, including these other types that are being swamped out by a predominant RNA species. That's the most optimistic possibility. To answer that question we need to use a homogenous RNA source, a cell type of known calcium currents. If we used one particular cell type that had a couple of different calcium currents in relatively equal amplitudes and then use that for the source of RNA, we know what to expect. When one uses rat brain it's not exactly clear what to expect in terms of the relative sizes of the currents.

Q: Ray Dingleline is also using whole brain messenger from rat and injecting it into oocyte looking for amino acid receptors coupled to channels. What he found was a chloride channel linked to an amino acid receptor which we don't see in vivo and I'm wondering if you see any such channel receptor linkages in your system. He thought that perhaps the chloride channel was endogenous to the oocyte which was linked to the messenger.

Leonard: Part of the reason barium is used is a preponderance of the calcium-dependent chloride channel in the oocyte. If calcium enters the cell through any means, this chloride channel is activated. Barium does not activate that channel to a large extent. That channel can be routinely blocked with DIDS or 9-ethylene carboxylate, but my guess is what he would be describing is some way of introducing calcium rise in the cell and getting an activation that is endogenous to calcium channel. It's the predominate blessing and curse of the oocyte; you can either use it as an amplifying method in the cell or it gets in the way and you have to block it. That's probably what he's talking about.

Q: Has anybody tried to take mRNA from the cDNA clone that Numa's group has on the dihydropyridine-insensitive calcium channel and injected into the oocytes?

Leonard: Yes, he had. The preliminary indications are that Numa has succeeded in getting a functional calcium channel activity from the one 70K protein, but there are controversies about whether in fact there are two high molecular weight peptides. One of these is a 175 weight glycoprotein. Numa indicates that they have evidence that only the one 70K protein is required to produce a functional calcium channel but that's at odds with other results. It is clear that the calcium channel has an S4 voltage sensor in it and a high-homology with 55% overall sodium channel, but functional activity has not been reconstituted yet.

Q: So that may indicate that something still is missing in terms of expressing that dihydropyridine receptor.

Leonard: With the proviso that there are other rat brain sodium channels, for instance, one of which has not been expressed; it's not clear that it's the channel's fault. It may be the oocyte's fault.

Michaelis: We're going to go from the expression of natural ion channels to a discussion of possible synthetic mimics of biological macromolecules and the following presentation is going to be "Polyammonium Macrocycles: Chemical Mimics of Biological Reactions", by Dr. Mathias Mertes.

Mertes: Being an optimist about the area and continually surprised about our results, I'm very encouraged about the applications that these molecules may have. Now, when we started this program about four years ago we asked the question, can we, as synthetic chemists, design synthetic enzymes? Or more properly, can we make more efficient catalysts? Why not take the catalytic features that are commonly found in enzymes and apply them in our synthetic model? What we're trying to do is mimic biological systems using the same catalytic features that are found in enzymes to promote reactions in macromolecules that are one-one hundredth the molecular weight of the natural systems. It's using more stable compounds, they're much smaller, much easier to design, and we can make them. What I'm going to spend some time on is our experiences with polyammonium macrocycles. The first consideration for us is can you readily make these molecules? Is there enough versatility in the synthetic methodology to change the functionality, vary the spacing, control the charge sites relative to one another? The second consideration is to apply enzymology to these systems and see if they work. We selected polyammonium macrocycles as our molecules.

I'd like to point out some of the advantages in polyammonium macrocycles. One of the key molecules is a 24 member macrocycle. One of the first considerations in making a macrocycle compound is that you can control its shape. Synthetic methodology based on old German chemistry is available to make these molecules, and while it's tedious, it's not difficult. The variations that can be made are illustrated by the number of analogues we looked at in this one study, and you can design these rather selectively, perhaps an asymmetric where you have ethylene spacing on one side and propyl spacing on the other side. You can also incorporate these aromatic groups if you want to change hydrophobic character or redox potential, and you can vary the hydrophilic-hydrophobic balance. Functionality can be introduced on these molecules either at the nitrogen or any of these defined positions on the carbons. Another advantage is that you can design not only monocycles, but also bicyclics, which we call bistren. If we're going to use these molecules to demonstrate biological catalysis, we need molecules that work in water. That consideration is very easy with these compounds because they're all ammonium compounds which, at pH7, are going to be protonated. You have water solubility and great pH control of the media because there's an internal buffer. Several examples of binding and catalysis, and two models for ion transport have recently appeared in the literature. A receptor for dopamine has been described which utilizes hydrogen bonding to the catecholamine portion of the molecule, and a crown ether for hydrogen bonding of the ammonium head.

The next example deals with selectivity for binding diammonium alkanes. The selectivity is dependent upon the length of the bridge between the two binding regions. In this case, you have a crown ether which has high affinity for the ammonium cation, and another crown ether. You can vary hydrophobicity and also the chain length. You could have selectivity based on chain length or the length of the tether groups. The next example is that of a dicopic receptor. The part which has a high affinity for carboxylate is bridged to a crown ether which has high affinity for the ammonium. There is a high affinity for binding omega amino acids. The length of the tether can be varied to get selectivity for the specific omega amino acid.

There are two papers by Lane and co-workers illustrating binding and catalysis. Reasonably high affinity is demonstrated for the ammonium head of bridged NAD analogs where there is a dihydropyridine NAD analog in the macrocycle. They observed binding and catalysis that well exceeded the bimolecular reaction for redox. There are two examples of carrier molecules that have been described. In a carrier molecule you need high selectivity and reasonably low affinity so you can have high exchange rates and proper lipophilic, hydrophilic balance. In this molecule, the tetracarboxylate was inserted into a lipid membrane. This is a pH driven reaction, acid on one side, base on the other side containing the metal. If the metal interacts with the crown ether in the presence of base, you're going to get the dicarboxylate. That's going to complex the metal in the cavity very strongly. As it equilibrates in the membrane, as soon as it encounters acid it's going to neutralize the carboxylates and transfer the metal ion. The recyclization back here becomes an equilibrium process with pH dependent transport of calcium above pH3. Dropping the pH below 3 results in a monocarboxylate with selectivity for the transport of potassium dependent upon pH.

The next example is a crown ether used for binding to an ammonium head in the macrocycle itself. This has a pendant ammonium head that's going to occupy the binding cavity as long as it's protonated. However, if it encounters a low pH basic media, it's going to be deprotonated, and that'll allow the metal to occupy the cavity and stay there until it encounters the acid. Once it encounters the acid, it will be protonated. This arm will be protonated and replace and release the metal. So you have a cation proton pump.

I'd like to present some of the results of two projects, one which is in progress and one of which we just started. The first will describe a chemical model for ATPase. The second will be a brief interlude into control and regulation, where coincidentally calcium, which is the second or third messenger, was found to affect the change in a reaction pathway in the super molecular complex. The third study is carboxylate activation of pH7 in water. And finally, a model where we are trying to mimic carboxypeptidase A.

The first study is the ATPase study. We are looking at the stability of ATP, which is stable in water of pH7 with a half life of about half a year. In the presence of ATPase it's about 0.22 microseconds. Nature has evolved a reaction that drives with an advantage of ten to the tenth. Can we utilize some of nature's catalytic events to drive the difference or at least enhance our control of ATPase hydrolysis such that we can approach even fractionally ten to the tenth? We did approach ten to the third;

better than non-catalytic, but we still have a long way to go. The general sequence of the reaction requires ATPases to go through a covalent phosphorylated enzyme intermediate. The first step is formation of a complex. If we're going to mimic this reaction, we want the complex wherein the chemistry takes place. The K_M is approximately ten to the minus five. This reaction is catalyzed by ATPase that is phosphorylated; a nucleophile on the enzyme attacks the terminal phosphate to give you the phosphorylated protein followed by water hydrolysis. Water is the second substrate to displace the enzyme from the phosphate and releases a second product and regenerates the catalyst. With the method for following the reaction that is a hydrolysis of ATP, we found very useful to follow the P31 NMR. For example, we used it with 24N502, the key molecule we're using today. At time 0 you have the typical peaks in the P31 for ATP. At 3.8 minutes there is formation of inorganic phosphate and ADP, and decrease in the other peaks. These should be equivalent. As the reaction goes on at 16.8 minutes, ADP is coming up, inorganic phosphate is coming up higher. At about 20 minutes it looks like we're about half life. Thirty-three minutes shows more ADP coming up, ATP disappearing. The method works well because you can program an NMR to work with your automated sequence. My acquisitions took about three and a half minutes. You can store these and two hours later gather the kinetic parameters. Another advantage is that you can now locate an unstable intermediate which is there only as long as ATP is present. It's a transient, unstable intermediate, and it was key to these studies. At first, I incorrectly assumed that it was pyrophosphate. We now find that is the covalent intermediate. Actually, we're transferring the terminal phosphate from ATP to the macrocycle and putting it on a nitrogen, resulting in a nucleophilic catalysis. Variations with the length of the chain show that when you have ethyl-ethyl-nitrogen or ethyl-ethyl-oxygen on both sides, they are the most efficient catalysts. For an asymmetric cavity there is no advantage, in fact, the activity fell. There is lower activity where you have the propyl cavities. The alternating ethylene diamine oxygen was not any good. When you put another group across which is identical to these two, you have what's called a bistren. Now the affinity of this compound for ATP at pH7 has a K_M of association of about ten to the eighth. However, catalysis is terrible. Our base molecule has a half life of eight minutes at a given temperature of eighty degrees. This has a hundred times greater affinity for ATP than the previous one, but the catalysis is twice as fast as water, which means it stabilizes. We have one molecule that stabilizes ATP. We want to look at that as a molecule that might be useful in making ATP from two molecules of ADP, if we can stabilize the product. It won't be much of a catalyst because it's not going to release the product.

Alterations that we employed recently to try to improve catalytic efficiency were to put lending groups on either one side or both sides of the molecule. We thought we'd put on a pendant amino group for nucleophilic catalysis as promoted by a lysine residue, or a serine, threonine, or cystidine. We looked at ATP hydrolysis with this and we didn't gain any advantage. When we restricted it by oxidizing the sulfide, we lost activity, so there is a high degree of structural specificity in this reaction that we don't understand. Interaction of the macrocycle occurred at pH7 where it's tetraprotonated. The

affinity for ATP is about ten to the fifth, which represents approximately a K_M of 10^{-5} . Hydrolysis of ATP can proceed by water attack of the terminal phosphate to release directly ADP and inorganic phosphate. Alternatively, the NMR indicated we had a phosphorylated nucleic macrocycle, so we made this compound and proved its structure by NMR and other techniques. We think, at least at pH7, the principle pathway for breakdown of ATP is through this phosphorylated macrocycle, then release of ADP, then water hydrolysis. The structure that we're depicting for this is just a working model; we have no evidence for this. We're illustrating binding of the alpha and the gamma phosphate to the two cavities. In this case we have a triprotonated and a diprotonated. It's more likely a diprotonated in both. You'd have to lower pH to about 3 to get a pendant protonated macrocycle. While this is based in fiction, the CPK modeling does fit using Sybil. You can see that the spacing is not bad. We folded this in a way that seemed like a fairly relaxed, low energy conformation. We haven't relaxed this in any computer program or minimization yet. If you see the CPK or the space filling equivalent, you can see its rather snug fit to the macrocycle and the gamma and the alpha phosphates hanging over the two cavities of the adenosine. As I pointed out, there is no evidence for this structure, but we have a starting model and we're trying to crystallize them. The overall sequence of the reaction is binding to form the complex. We're depicting nucleophilic attack by this nitrogen on ADP, its terminal phosphate, in an additional elimination mechanism. This gives the phosphorylated intermediate disassociation of ADP from the cavity and the interaction with water to regenerate the catalyst, which starts over again with ATP. The enzymatic reaction is reasonably good, and we were quite pleased with the affinity. Acid catalysis is clear. In covalent catalysis, we see the additional elimination reaction. Water hydrolysis of the phosphorylated intermediate is also present. Product association, certainly, in metal catalysis, which we'll talk about in a minute.

We looked at the unstable intermediate at about ten parts per million. When we took the same reaction media at pH7 and one to one equivalent of calcium bromide, one calcium, one ATP, one macrocycle, we saw two things that were quite interesting. First is that this is an unstable intermediate. Look at the amount in the presence of calcium compared to what we saw before. The maximum amount ever achieved in a straight ATP reaction is about 12%, and we're getting close to 50% phosphorylated intermediate from this reaction. So you change either the stability of the intermediate or the speed with which it breaks down ATP. It's twice as effective as a catalyst in the presence of calcium, but more importantly, you have a lot of the phosphorylated intermediate. When we followed the reaction we saw the same pattern: ATP disappearing, ADP coming up, and a large amount of inorganic phosphate; as we let the reaction go on, we saw another peak coming up. At the end of the reaction, there's still a little bit of ATP running in there. Phosphorylated macrocycle is essentially gone but we have this new peak. The peak assignment was identified by spiking it with pyrophosphate. We took ATP and ran it through an intermediate phosphorylated macrocycle and in the presence of additional inorganic phosphate, caused a reaction in the cavity to form pyrophosphate at pH7. Pyrophosphate is an anhydride and a rather high energy molecule. We were rather surprised to see that result, but are not sure why this

happens. Three things must be explained: why is it a catalyst, why does it form so much phosphorylated intermediate, and how do you get pyrophosphate? We think that calcium, like magnesium in the natural system, orders the substrate and makes the reaction. The binding constant has not been determined, so I don't know that it's not binding or affecting affinity. However, if you order it a different way, with beta gamma binding to the calcium, as we look at the NMR of these carbons, it turns out that the greatest effect on the presence of calcium on this system was on the CH₂'s adjacent to the oxygen. So that's an indication that there's a change in the dihedral angle, and the electrical static field around the oxygen. Why the phosphorylated intermediate? We presume this other cavity is open and the occupancy by inorganic phosphate causes a reaction, a nucleophilic attack, to displace the PN bond. What we've done is added a third effector molecule. In the absence of calcium the primary reaction runs down and up to ADP. In the presence of calcium we enhance the reactivity in this direction and also form a new product, pyrophosphate. So we've effective control of change in the direction of the chemistry in this macro supra molecular complex.

The next study I'd like to talk about is carboxylate activation. Carboxylate activation in water is a very difficult process. You've got the anion hydrogenated, and to get any kind of reaction at carbon would normally require nucleophilic attack at the carbon. This has a large anionic shell and biology takes care of it by forming a product with a good leaving group on that calcium. For example, glutamine synthesis: glutamate, is phosphorylated on the terminal carboxyl, and the mixed anhydride is reacted to the amino equivalent to yield glutamine. Other examples of carboxylate activation in protein synthesis are to form the mixed anhydride with ANP and the amino acid. Biology activates carboxyls by forming a highly reactive intermediate with a good leaving group, subject to nucleophilic attack. A more pertinent example is that of formate in the presence of ATP, tetrahydrofolate and magnesium; in this case called n-10-formyltetrahydrofolate. You're taking an anion in this aqueous media and forming an amide. To do that in inorganic chemistry, you have to make an acid chloride and an ester, and make a reasonably high energy intermediate. Normally one would go to a nonpolar solvent and heat it up. Biology does it very effectively at 37 degrees, but one of the key questions is that the intermediate in this reaction is the mixed anhydride formed from the energy of ATP hydrolysis. You activate this carboxyl by making a mixed in hydride with phosphate. So, we looked at formyl phosphate, and tried to examine some of the effects of our macrocycles. Acetylphosphate in water and in many enzymatic systems breaks down by PO bond cleavage we end up with a phosphorylated macrocycle. If we get CO bond cleavage, we should formylate the macrocycle and release inorganic phosphate. In both cases, this would break down to inorganic phosphate. However, we should be able to detect the difference because that amide should be stable and a formate should be evident.

We look first at the effects of this on hydrolysis. Formyl phosphates are rather reactive and difficult to keep around. There is a water catalyzed hydrolysis of formyl phosphate which runs at about pH7. The catalytic effect is about 400. So it greatly enhances the breakdown, but it's not a hydrolysis reaction. We followed the

reaction with NMR. In pursuing the reaction, we found that the product of the reaction was breakage of the carbon phosphate bond and formation of the formylated macrocycle. Given that, with formyl phosphate, can we utilize our system in the activation of formate? We took the reaction mixture of ATP, calcium, and formate, and mixed them. The ATP goes down, the PN bond comes up, phosphate comes up and ADP forms. We added formate anion solution, formic acid. We took the carbon spectra after several hours and found the peak for the formylated macrocycle. ATP initially is going to give you a rather high yield, in the presence of calcium, of the phosphorylated macrocycle. ATP is required for this reaction. The addition of formate is thought to give binding to this cationic hole, which then can subsequently attack the phosphorandate just like pyrophosphate did before to give us an intermediate formyl phosphate. Formyl phosphate is very unstable and would immediately go to formylate the macrocycle with its release. So we feel we have a model for activation of formate. When you think about the reaction, we're really dealing with three bonds that are formed and three bonds that are broken with the energy supplied by ATP in discrete steps. Basically, you have the complex formation which overcomes this barrier to give you the phosphorylated intermediate, NADP. You get exchange of the ligands of the other cavity where you have dissociation, reformation of the phosphorylated macrocycle complex with formate. This goes over the barrier to give you the intermediate formal phosphate, NADP. This followed through to give you the formylated macrocycle, an inorganic phosphate which then disassociates. So you start out with the macrocycle formate NATP, you end up with formylated macrocycle PNADP.

The next part of this talk is about the chemical model for carboxypeptidase. The catalytic features of this enzyme for hydrolysis of esters are well known. Arginine 145 is thought to be a binding site for the terminal carboxy. Glu 270 is thought to be a nucleophile, at least in ester hydrolysis at the reactive center. Zinc plays an important role, both for polarization of the carbonyl, to enhance reaction at this point, and also for activation of general base catalysis, and activation of water or OH⁻ for subsequent attack at this point. The intermediate in ester hydrolysis gives the Glu 270 anhydride. Much like the cholinesterases or the serine proteases, you have acylation of the enzyme and then breakdown of that catalyzed by the water. Can we design an enzyme mimic for carboxy peptidase that may be useful for cleavage of carboxy terminal peptides? Using aromatic groups which will not be protonated at pH7, you should be able to use complex metals such as zinc. At pH7 these will not be protonated, although they should be. At the Arg 145 binding site for the terminal carboxy, the zinc should polarize the carbon that is going to be attacked. There is the Glu 270 equivalent, sitting off to the sides directly in line of attack of the carbonyl carbon. Next is synthesis. This is a retrosynthetic approach. We have an asymmetric system that's created several problems. After breaking this down in a retrosynthetic way, our cleavage point is going to be on one side. One side will be the western half of the molecule which is formulated as the diamide amine. The right hand side is constructed stepwise. The intermediate is activated for displacement by the amine. To get to this system, we tried to build it up one nitrogen at a time. At one point, we got a mixture of the three end products and the four end products which we

could not separate. So we broke it at one point, making the bottom piece first and reacting with the anion. Right now we have achieved the protected nitrile; we have tosyl groups there protecting and we have puridine derivative. So the next step is removal of the tosyl groups. The question is, do we have from the nitrile an amide or a carboxylate. If it's a carboxylate we're in trouble because you have an amino acid. We want it to be the amide at this point just for purification purposes. We've found a rather high affinity for binding substrates and have selectivity based on charge density. You also can employ steric effects, and the distances between the charges to achieve selectivity. We've demonstrated catalysis and we've seen regulation by the addition of calcium to change the chemistry of the product.

I'm excited about the many possibilities in application. Long range goals are that we want to continue to create receptors. We want catalysis for synthesis or degradation of rare molecules. There is some work going on right now on ion channels. Lane has described a rather strange molecule, a macrocycle with a lot of hydrophilic appendages. I'm afraid they're going to stack the wrong way in the membrane and make internal transport possible, but not transmembrane transport. The challenge now as I hear it today is to try to make a receptor that is coupled to an ion channel, or a transport molecule that can be modulated by a third effector molecule.

Q: Concerning the macrocyclic experiment with the ATP, is there free energy? Obviously, you said you'd do an additional elimination reaction, and go through some sort of transition state structure. It would be nice to look at the energetics on binding to a macrocycle. You can do that if you figure out a catalytic assay.

Mertes: We have the association constant, but I don't really know how we can get at a transition state.

Q: Do you have any evidence for the inversion of that phosphorase?

Mertes: Not at all.

Q: The other question is in the design concept of your carboxy peptidase A. Taking into account that in the actual enzyme mechanism you're going to go through, zinc goes through a penta coordinated intermediate. You may have allowed for that in your structural design.

Mertes: Zinc is not going to gain a great deal of energy by changing coordination shells.

Eldefrawi: I noticed that you used carrier molecules. And definitely, macrocyclics that act as carriers are among the natural products the fungi, bacteria and a large number of organisms would use. Now carriers are also very important biological regulators, as the major mechanism of terminating neurotransmitter action is through uptake that utilizes transporters or carrier molecules. Because they are the targets of many incapacitating agents and very important drugs - to mention a few, cocaine, PCP, and tricyclic antidepressants - we should give them a lot of attention. Now, would you care to comment whether

there is any progress at all towards making some of these synthetic carriers?

Mertes: Nobody has done anything as far as I know. One point I should mention is the advantages. There shouldn't be any problem in use on solid surfaces.

Michaelis: We're going to close today's session with the last presentation, and this time the emphasis will be in the other type of cholinergic receptor, the muscarinic receptor. The presentation is, "Structure and Function of Muscarinic Receptor Subtypes", by Dr. Ramachandran.

Ramachandran: I'd like to thank Drs. Michaelis and Valdes for inviting me to participate in this symposium and to discuss some of our recent work. Intercellular communication is accomplished not only through the neurotransmitters, but also through secreted hormones and growth factors to the specific interactions of these agents with receptors on the target cells. Invariably, these receptors are complex integral membrane glycoproteins, present in extremely low abundance except for the much discussed nicotinic acetylcholine receptor. The arrangement of these receptors in the plasma membrane of the target cells is dictated by the topological distribution of the hydrophilic and hydrophobic domains. If one has information about the primary structure, the amino acid sequence of these receptors, one can begin to understand aspects of the mechanisms by which transmembrane signalling is accomplished. Although a great deal has been learned in the last two decades through studies using ligand binding, usually kinetic characterizations of these receptors, this is not sufficient for understanding the molecular basis of signalling; and structure, of course, is the key to this. Until recently, it was not possible to even conceive of the structures of these receptors except in the case of the nicotinic acetylcholine receptor. The extremely low abundance and the very hydrophobic nature of these receptors precluded isolation and characterization by conventional approaches. However, with the advent of recombinant DNA techniques, this has now become feasible. In the last three to four years, we, as well as others, have succeeded in cloning the genes coding for several of these receptors. The approach that we have taken is a straight forward one; namely, to purify the receptor proteins, obtain partial amino acid sequence information by microsequencing procedures, and to design probes based upon this information. We then screen suitable cDNA libraries and obtain the clones from which the DNA sequence is obtained, and deduce the amino acid sequence from the nuclear type sequence. As a result of the kinetic studies, we have a great deal of information about the receptors. Almost all of the cell surface receptors that one deals with can be put into three classes. The most discussed one at this conference is the one representing the ion channels. We have ligand-gated ion channels such as the nicotinic acetylcholine receptor and GABA and glycine receptors and voltage-gated channels.

The second class of receptors is the growth factor receptors. The characteristic of this group is that the ligand binding domain and the effector domain are part of the same molecule. The third class is the one in which the signal is transduced via guanine nucleotide

binding protein. The receptor is activated by the ligand and this interaction of the G protein with various enzymes leads to the generation of the second messenger. Recently, we succeeded in cloning the GABA_A receptor in collaboration with Professor Barnard and his colleagues at Cambridge University. These do not directly relate to the ion channels, but we have cloned the insulin receptor, the IDF-1 receptor and EDF receptor. Most recently, Genentech and UCSF have cloned a PDGF receptor, and the main feature of this is that they all have a single transmembrane domain where only the hydrophobicity is concerned, but not the amino acid sequence. The ligands bind to extracellular domains. We have learned a great deal just from the sequence analysis of these molecules. It is remarkably well conserved across the receptors, and the specificity of the function depends on the intercellular substrates that are acted upon by these kinases. We have now expressed the human insulin receptor in rat fibroblasts at quite high levels of about one million sites per cell, and are able to isolate milligram quantities of the receptor. We obtain material in sufficient quantities to begin the kind of structural and physical characterization that Dr. Hess was talking about, so I'll move on to the third class. A variety of aminergic and peptide ligands interact with the receptors, and either activate adenylate cyclase through guanine nucleotide binding proteins or inhibit cyclase activity through the inhibitory guanine nucleotide binding protein. These G proteins are heterotrimerous, composed of an alpha, beta and gamma subunit which stay together. Often it's the alpha subunit that is activated, which then acts upon the adenylate cyclase either to activate or to inhibit it. It has become apparent in the last couple of years that the turnover which is catalyzed by the phospholipase C is also mediated by a different G protein, which some people refer to as GP. A variety of hormones activate this pathway and then, with the hydrolysis of phosphoinositide, this phosphate goes to diethyl glycerol, then leads to the activation of protein C kinase and mobilization of calcium from intracellular pools from endoplasmic reticulum which leads to the generation of the second messengers.

We investigated two receptors in this group, the beta adrenergic receptor, as an example of the receptor interacting with the stimulatory guanine nucleotide binding protein, and the muscarinic acetylcholine receptor. Both are examples of the inhibitory pathways as well as the activation of the phospholipase. In addition, the muscarinic receptor activates the potassium channel. The subtype diversity of the muscarinic receptor has been very interesting and challenging, and will be illustrated when the binding of an antagonist such as QNB to muscarinic target tissues is competed with atropine. There is no distinction between the various subtypes in different tissues. However, certain drugs such as pirenzepine distinguish between the subtypes in different tissues. The molecular basis of this subtype diversity has been debated for a number of years. Several people favored the view that they represent different conformational states of the same receptor, or different lipid environments if found in different target tissues. They were structurally different, either both translationally or otherwise different. We embarked on this project to clone this receptor as a means of understanding the molecular basis of the subtype diversity. We did this by working on the porcine atrial muscarinic receptor which, according to

pharmacological criteria, has been classified as an M₂. It binds pirenzepine with low affinity. We obtained affinity purified muscarinic receptor. The purified receptor then was subjected to amino acid analysis, and like all G protein-linked receptors that have been studied to date, the amino terminal had to be blocked. We had to then generate peptide by trypsin treatment of the purified receptor. This generates the muscarinic receptor from 80 kilodaltons, and this cleaves to a 50 major fragment and several small peptides of less than 6 kilodaltons. We could fractionate these on the HPLC. These four peptides then were sequenced and these sequences were used to design all the nucleotide probes which were used for screening. First a porcine genomic type library from which we obtained the clone appeared to contain all four peptides that were obtained here. Then the restriction fragment from this clone was used to screen a porcine HPLC-DNA library and we were finally able to obtain overlapping clones which coded for the entire receptor. I have the complete amino acid sequence deduced from the nucleotide sequence of these clones. The end terminal is blocked. This was a sign that the initiation site was based on the nucleotides which meet Kosak's criteria. By this time it was already known, since the beta adrenergic receptor was cloned earlier, that the coding sequence for the beta adrenergic receptor was present on a single exit. There were no entrances in this region. It appears that the muscarinic receptor is also similar in that when comparing the genomic clones and the cDNA clones, they could tell that the entire coding sequence is present in a single exon. Unlike the beta adrenergic receptor, we found alternative splicing in the 5'-prime translator region. There are actually two different exons, exon 1A and exon 1B. These are in the untranslated region and these are the various spliced donor sites and the acceptor sites. There are termination codons before reaching the initiation code, so the coding sequence is the same for all types of splicing. In screening a prime library, it turned out that exon 1B was expressed in a ratio of 8 to 1 over 1A, so there is a preferential splicing and this may have something to do with the expression levels in different tissues.

Our idea was to obtain the amino acid sequence and perform a hydrophobicity analysis to discern the hydrophilic and hydrophobic domains. In the case of the heart muscarinic receptor which we cloned, you find, unlike the growth factor receptors, several hydrophobic domains which qualify as transmembrane regions. One can clearly see such domains. Now, by analogy with the beta adrenergic receptor for which the assignment was made, visual rhodopsins also interact with the G protein. This less hydrophobic but neutral region was also the same as the transmembrane domain. We worked on the porcine atrial receptor while Numa and collaborators were working on the brain muscarinic acetylcholine receptor from the porcine species. When we had cloned the turkey erythrocyte receptor, the hamster beta 2-adrenergic receptor was cloned by Lefkowitz and the Merck group. Although there were significant differences, we could not attribute these differences to the structural differences between what is known as beta 2 pharmacologically, and that which qualifies as a beta 1. These subtypes are due to the structural differences of subtype nature, the species differences. By comparing the sequence that was deduced by Numa's group, we could immediately tell that the subtypes are coded by separate genes. Based on the hydrophobicity analysis in comparison

with the beta adrenergic and the rhodopsin receptors, this is the orientation of the muscarinic acetylcholine receptor that we favor. There is a sequence at the end terminal and potential glycosylation sites. The molecule is easily glycosylated, so all three are probably involved. The receptor weaves through the membrane seven times. The similarity between the brain and the heart receptors for the muscarinic receptor is very dramatic. There is only 38% amino acid identity between the two receptors derived from the same species, so it is clear they are coded by separate genes. The similarity that remains between these is primarily in the transmembrane domain. The transmembrane domains are heavily conserved, and in the two cytoplasmic loops the first and the second small loops are also reasonably well conserved. What is striking is the third cytoplasmic loop which is very large in the muscarinic compared to the beta adrenergic receptor. In rhodopsin it's extremely small, only about five or six residues. There is little identity between the porcine brain muscarinic and the porcine heart receptor; there are hardly any similarities in this region. This similarity suggests that this may be important for differential coupling of the receptors to different signals. For example, in the heart, the primary effect of acetylcholine seems to be to inhibit adenylate cyclase, and in the brain it is stimulation of phospholipase-C. Perhaps these regions have a role in that, but no one can verify this by performing deletions in mutants in this area. Such studies are already underway on the beta adrenergic receptor.

The other interesting feature is that, unlike the growth factor receptors, there is a single hydrophobic transmembrane domain and no charged residues are present. The transmembrane domain in the G protein coupled receptors contain charged residue. For example, there is an aspartic acid in the second transmembrane domain, and another aspartic, and these are fully conserved both in the muscarinic and the adrenergic receptors. These residues probably are important in ligand binding, especially in the case of these amine type of ligands. The other feature I'd like to point out is that this third cytoplasmic loop in the brain receptor has 156 residues. The heart receptor has 180 residues. The brain receptor contains several potential sites of phosphorylation. In cyclic A kinase three such sites are completely missing, but there are others which are potential sites of phosphorylation. Now it has become apparent in the last few years that the desensitization that several of these G-protein receptors undergo is linked to phosphorylation of the receptor. These receptors undergo phosphorylation in an agonist-dependent fashion. Even rhodopsin undergoes phosphorylation in a light-dependent fashion, and this leads to desensitization. The interesting thing is that the phosphorylation of regulatory sites are different in the brain and heart receptor. This is interesting and encouraging in terms of drug development because one can design selective antagonists for these potential kinases which may affect the regulation of one receptor but not another. In a sense, just learning the structure of this has pinpointed new potential protein targets for drug development.

All of this information was developed simply by looking at the structure of the amino acid sequence of these molecules. What is the proof that we have cloned the proper receptor? We have expressed this M₂ porcine muscarinic receptor in Chinese hamster ovary cells. The muscarinic receptor gene is driven by a promoter and we have an HIV

long terminal repeat and then a gene for selection and amplification. When Chinese hamster ovary cells are transvected with this plasmid and selected, one can obtain stable cell lines expressing very high levels of these receptors. We have characterized the binding of the recombinant receptor in these transvected CHO cells, and there are 1.5 million sites per cell in this system. The recombinant receptor shows the proper pharmacological specificity that is expected of an MA₁ myocardial receptor, namely atropine, which binds with a high affinity. Analysis of the porcine genome, and later the human genome, showed that it's primarily coded by a single gene, but we did find other weakly hybridizing bands. By this time, we clearly understood that the subtypes are coded by separate genes and that other subtypes may exist. We then screened a human genomic library to obtain the human counterparts of these muscarinic receptors and the logic here is that, like the beta adrenergic and the porcine muscarinic receptors, it is very likely that the human muscarinic receptors are coded on a single axon. A genomic library was sufficient for this and we were able to obtain them using a 680 base parafragment, corresponding to the first five transmembrane domains and the two cytoplasmic loops, which are well conserved. We were able to, by hybridizing at low stringency, pull out 23 clones and classify them into four distinct classes. Using the information that the third cytoplasmic loop between the fifth and sixth transmembrane domains is unique to the M₁ and M₂ subtypes, we used probes from this region to identify the human subtypes. We were able to obtain not only the human M₁ and M₂, but also two others which we called M₃ and M₄, which are structurally related to the other two. This is simply the restriction map of the four human muscarinic receptor subtypes that we obtained. All four are sequenced and have the structures compared. They all have the same transmembrane topology, and comparing the sequence of what we call HM₁, human muscarinic 1, with the porcine muscarinic 1, there is 98.9% amino acid identity. Even though between the two subtypes in the same species there was little identity across species, the subtypes have remarkable identity. The HM₄ is the largest of the four. These are all usually 460 to 480 amino acids, but this one is 590 amino acids and has a huge cytoplasmic loop and also a larger interterminal sequence. HM₄ and HM₁ are the most closely related on this basis. You do see certain number of messengers conserving the large cytoplasmic loop. HM₁ is the residues that are conserved in all the muscarinics and the beta adrenergic receptors. The similarity between the HM₂ and HM₃ is closely related. HM₁ and HM₄ are related, and M₂ and M₃ are related. It's clear that the aspartic acids that I mentioned earlier in the transmembrane domains are all conserved in these molecules also. In order to verify the properties of these cloned human muscarinic receptors, we have expressed them transiently in U293 cells. This is a human kidney carcinoma cell line, using different expression system, and it shows the pharmacological properties that are known for the various types. For example, the M₁, atropine, binds well to all four as expected, but pirenzepine has high affinity for M₁ and AFDX116 has low affinity for M₁. The opposite is true for M₂. Pirenzepine has low affinity and AFDX has higher affinity. The other two fall in between, and turn out to be similar to M₂ and M₃. M₁ and M₄ are more closely related. These are antagonist, binding carbachol, and each subtype now shows multiple affinity states. It has been known for a number of

years that there are a number of affinity states. It could not be attributed to a single receptor subtype existing with a high and low affinity state by interaction with a G protein because these tissues were always heterogeneous. Many of the properties attributed to the brain receptors which are thought to be M_1 are probably also M_2 and M_4 as I'll show from organ analysis. The AFDX116, which is thought to be an antagonist, is actually acting as a partial agonist, giving rise to low and high affinity states. In some other subtypes, for example HM_1 and HM_2 , only HM_1 acts as a pure antagonist.

An organ analysis was then performed in order to assure ourselves that the two subtypes were also expressed in normal tissues. The tissues that were probed were whole brain, pancreas, severed cortex, heart and this NG108 neuroblastoma cell line. When these are screened, it's apparent that whole brain contains all four subtypes. In the heart, M_1 , M_2 and M_4 are not expressed, but M_2 is very clearly seen. Heart seems to be essentially M_2 , although there may be some low level of expression of the others. The pancreas doesn't seem to have any of the first three, but we can see expression of HM_4 . This glandular muscarinic receptor subtype can be distinguished by low affinity for AFDX116 from the heart which is high affinity. This appears to be HM_4 , and interestingly, NG108 seems to have exclusively M_2 subtype. In addition to having the different subtypes localize to discrete parts of the brain, it is likely that, even in a given region of the brain, neurons may be expressing unique subtypes.

We have proceeded to characterize these receptors in more detail, because the next important question for us is whether these receptors are coupled to unique biochemical mechanisms. It has been assumed that the M_2 subtype inhibits adenylate cyclase. Having these pure subtypes, we are in a position to examine them by a chemical coupling mechanism. We have done this using a porcine M_2 . The previous data were from homogenate since we were going to be working with intact cells. The apparent K_d was 75 picomolar. When we do dissociation kinetics, we get 78 picomolar, in excellent agreement with what has been obtained with purified preparations of this receptor. Next, we examined the binding of the agonist carbachol to membranes prepared from these recombinant receptor expressing cells. We could then verify that multiple affinity states exist for a pure subtype. The Chinese hamster cells are very good for this work because they seem to have no endogenous muscarinic receptors. There must be less than 100 per cell, and therefore we see no binding in those cells. The transfected cells, in the absence of any GTP or other treatments, show a high and a low affinity for carbachol. This can be abolished in the presence of GTP γ S or with treatment with pertussis toxin which uncouples the G protein from the receptor. Therefore, one doesn't get the high affinity state one sees with muscarinic receptors. It can derive from a single pure subtype owing to the interaction with the G proteins. We looked at cyclic AMP inhibition, which is characteristic of the myocardial muscarinic receptor, and we saw that carbachol stimulates cAMP inhibition which is transfected with the muscarinic receptor gene. One gets 50% inhibition at 7×10^{-8} molar carbachol. What was surprising was when we examined the turnover of PI in the same system, we found that carbachol could stimulate this response also, although at high concentrations. However, both responses were antagonized by atropine and pirenzepine with the same affinity, again

showing that the two responses to carbachol are mediated by a single pure subtype which we have transected into the CHO cells. This can happen in two ways; one is that the muscarinic receptor is able to couple the two mechanisms used through a single G protein with separate tracts with guanine nucleoprotein. Perhaps we are able to force the coupling to PI turnover. The other possibility is that there are two different G proteins mediating the two responses. This expression has enabled us to force the muscarinic receptor, which normally wouldn't couple to PI turnover, to couple in this system. We examined a number of cell lines which are selected to have different expressed levels of receptor. The Chinese hamster ovary cell Y type has been transected with OKT4, and it shows no response to carbachol in either PI turnover or in cAMP inhibition. Then, cell lines which express 2050 sites per cell, 600,000 sites per cell, and 1.5 and 2.5 million sites per cell were chosen. The PI turnover is actually closely linked to the receptor number, and only reaches saturation at about 1.5 million sites per cell. On the other hand, cAMP inhibition seems to be essentially independent of the number, reaching saturation around the same concentration in each case. This would immediately suggest that two different G proteins must be involved in the coupling to the two responses. To verify that, we then examined the sensitivity of the two responses to inhibition by pertussis toxin. Pertussis toxin is primarily known to affect the alpha subunit of the inhibitory type protein. That would uncouple the cAMP inhibition response, which is very sensitive to pertussis toxin. One nanogram per ml completely wipes out the carbachol-induced cAMP inhibitory response in these recombinant cells. On the other hand, the PI turnover requires a much higher concentration of pertussis toxin to abolish this response. In the brain, PI turnover is not sensitive to pertussis toxin, so the G protein that is mediating the PI turnover in the CHO cells is probably of a different type. In order to further verify this, we looked at the alpha subunit, which has a molecular weight of 41K. If you didn't treat it with pertussis toxin, the radioactive NAD does not label the alpha subunit, but if you treat the membranes, you get label. If you pretreat the cells with pertussis toxin then the subsequent treatment with toxin, label does not affect the subunit. One can use this assay to measure the amount of pertussis toxin substrate that is remaining after various treatments of the cells with different concentrations of the toxin.

I would like to finish by pointing out certain features in these molecules, unlike the growth factor, receptors where we have been able to discern the orientation of the molecule but have no idea of what the molecule looks like. We have a very good model for the G protein coupled receptors because of the extensive information available both in visual rhodopsin and bacterial rhodopsin. The orientation of the transmembrane domains in bacterial rhodopsin from diffraction studies shows that the seven transmembrane domains are oriented as a cylinder. This is likely to be the arrangement in the case of the adrenergic and the muscarinic receptors. The ligand then binds into the pocket and now it's understandable why there are charged residues. Aspartic acid in the second and the third transmembrane domains may be involved in interactions with the quaternary ammonium, and then the other interactions may stabilize this binding. The ligand may lock-in this kind of a conformation and that may be the axis of the

large conformational changes that are induced into these molecules by ligand binding. These things can be readily verified by mutating out the aspartic acid in the second or third transmembrane domain and showing that the ligand binding is affected. Although we have been talking primarily about the adrenergic and muscarinic receptors dealing with amine type ligands, the peptide hormone receptors are also likely to have such an orientation. So far none of the peptide hormone receptors mediated by G protein have been cloned, but this information is already available in the literature from yeast. In yeast there are two types of cells, the alpha cells and the A cells. The mating of these is controlled by pheromones, called alpha factor and A factor, which turn out to be small polypeptides about eleven and twelve amino acids. A cells have alpha factor receptors and alpha cells have A factor receptors. By complementation, a Japanese group and a group in this country have been able to clone the genes coding for these two receptors. They based it on the amino acid sequence, and analysis appear to have seven transmembrane domains. This looks like a very ancient, very useful mechanism that has been utilized by nature for signal transduction. Although it needs to be proven, it is likely that some of the peptide hormones that mediate responses to G proteins may contain this kind of a topological arrangement of the receptor.

Q: When integral membrane proteins, like receptors, are expressed in cells, about what quantity of protein you are talking?

Ramachandran: Yes, we were originally very concerned that the seven transient brain domains proteins may not be expressed and it would distort the memory. It turns out to be easy. They seem to love to go into the membrane. We have selected cells which can express up to six million sites per cell, so when we have one million sites per cell of the insulin receptor, I'm able to get a milligram, which is about five nanomoles of the receptor. Although the yeast system has great advantages, one can turn to mammalian cells as an expression system, even though it's more laborious and requires perhaps more special set up. I will qualify this because one of the interesting features is in the growth factor receptor that is a single transmembrane domain. People are postulating that the single transmembrane domain involves aggregation in the plane of the membrane. These G protein coupled receptors have the seven domains which are already linked, and it looks like opening and shutting. They probably have no problem expressing functionally. I don't know what will happen in the multisubunit ion channel type of receptors, as we haven't expressed any of those in the mammalian cells.

Q: What percentage of the membrane protein ends up to be the beta adrenergic receptor when it's expressed in the cell?

Ramachandran: In natural systems?

Q: No, in the CHO. Do you end up with almost all the protein there?

Ramachandran: No. We're getting in the range of 0.1 to 1 percent depending on the level of expression. Normally we are dealing with 1000 to 5000 sites per cell. A hundred roller bottles of the rat

fibroblasts which are expressing insulin will give me a milligram in two weeks.

Eldefrawi: We are interested in muscarinic receptors and their possible interaction with anticholinesterase nerve agents. Muscarinic receptors are extremely susceptible to the action of nerve agents. The system that you have developed with transfectant cells seems the ideal system on which one would test the effect of these agents. Have you looked at kinetics of binding?

Ramachandran: Those are important studies, but no, we haven't done those. You can see that's a small group handling four different recombinant receptors. We're trying to sort out the coupling mechanisms. We are showing the M_2 couples primarily to the cyclase inhibition. We are in the process of doing the human M_1 , to see if it's primarily coupling to PI turnover. It looks like it. And we want to see if the reverse is true, that over expression will push it to couple with the cyclase inhibition as well. The question is, what are the couplings to the potassium channel?

Michaelis: Are there any questions that anybody would like to raise from any of the members on any aspect of the presentation?

Q: With respect to this morning's first presentation, I was wondering why it wasn't practical to look at activity on cholinesterase per se, rather than looking at activity on the channel which requires the presence of cholinesterase or the activity to be observed.

Valdes: We did not disregard the possibility that you may get some activity with the esterase. We assayed for the anticholinesterase through a receptor response rather than through esterase inhibition. We thought it a more powerful, sensitive tool, because if you look at the cell response and monitor the anticholinesterase's effect through the response of a receptor, it is at least one to two order of magnitude more sensitive. At the time you see 50 percent or 80 percent inhibition of esterase functioning. If you do a spectrophotanalysis, you are looking at a five to ten fold increase in the receptor response, so instead of reducing your enzyme activity by half, you are increasing your receptor response by five to ten fold. We followed the receptor response by monitoring the binding of PCP at a certain period of time as a receptor response cue. That increased from almost one or two percent activity to 100 percent activity. At that concentration you have reduced the enzyme activity by about 90 percent or 95 percent. So, if you look at the increased receptor response, it is a lot more sensitive. When we came to the application in the initial stage, we decided to monitor the receptor response. We want to use a system that simulates a cellular situation and follow the effect of DFP on the receptor response, rather than just esterase inhibition.

Q: The experiment you described seems as if it would be related to stoichiometry. If one were to bind cholinesterase on the surface, one might instead end up with more comparable or better sensitivity. Do you have any feeling for the relative binding constants?

Eldefrawi: You are reading my thoughts of several months ago. We are considering that we ought to be titrating the systems now, not just how much lipid for every receptor site, not how much receptor site for esterase site or how many lipid molecules; all these quantitative aspects are on the board and what you said is absolutely true. We did this on the test tube pharmacology—that the ratio between catalytic sites and the receptor sites, are important to the magnification of the signal. We are still at step one, but these are very significant questions, and answers to them will tell us how to come up with the best possible product.

Q: If you take isolated subunits in which acetylcholine or other binding sites are on specific subunits, does the subunit have to be orientated properly in the membrane? Does my question make sense if you get a binding specificity on an uncoiled subunit on which the proper amino acids are present?

Eldefrawi: The dodecapeptide of alpha subunit acetylcholine receptor will bind bungarotoxin. It's in the affinity that is about three or four orders of magnitudes less than the protein, but still it has a dissociation constant of 10^{-8} . It appears that only a very small sequence of one of the subunits is sufficient to interact with toxins.

Q: If it mimics the chemically synthesizing parts of receptors, that would help overcome the stability problem, because many of these receptors aren't that stable with which to work.

Michaelis: Let me reiterate here now that in addition to what Doctor Hess said, the work of Karlin and his colleagues of trying to label specific sites for drug binding on the acetylcholine receptor indicated that the folding of the peptide is very important to how the drug binds. It is not just a fragment that constitutes the very high selective affinity that we look at. You require other amino acid residues that come in from folding of the peptide in a particular shape. You lose three or four orders of magnitude in the affinity of the ligand. If you have a primary structure of the peptide, you may still bind, but with very low affinity that may destroy the whole purpose of making a biosensor that would have very high recognition capability. You would like to get it in the best possible conformation, and for that I maintain that you have to go into a bilayer and take its natural folding so that you maintain that recognition capability.

Q: If you have to put the subunit into a lipid bilayer to fold it up correctly, do you need all of the multiple subunits? For example, in acetylcholine, can you just put alpha subunits in a lipid bilayer and get nearly the same affinity that you would get if you had the alpha, beta, and gamma, delta subunits?

Ramachandran: If you deal with a single subunit you are going to lose some affinity, because the other subunits contribute to conserve the folding of one subunit by influence of the other. However, there is no way to tell without experimenting. The single subunit would probably give some response. It may be a response weakened to the point of a

loss of two or three orders of magnitude again. So, one would have to balance the benefits versus the losses; if the benefits are that you can get a small dodecapeptide like Dr. Hess suggested, it would have recognition capability, but we can synthesize it in the laboratory in gram quantities and sacrifice the sensitivity. But if sensitivity is very important and the selectivity of recognition is very important, one may have to go to the much larger protein.

Q: It appears that a single subunit of a protein can have both unique characteristics and all of the properties of the ligand binding recognition; for example, the muscarinic receptor.

A: Yes. The problem is that you want selectivity. This analysis tells us that the ligand binding domain for many receptors is similar and one or two residues are making the difference; beta adrenergic ligands bind to adrenergic receptor and acetylcholine binds to the muscarinic. Selectivity and specificity are the keys, and for that you need the specificity that is built into these multi-transfer membrane domain systems.

Broomfield: There is an implicit assumption in this discussion that simple binding is going to give a signal. If ion channels are to be formed, then I think you have an entirely different problem. I doubt that simple binding is going to give you very much of a signal.

Eldefrawi: I'm not so sure about that. If you read quantum mechanics and you follow up what's supposed to happen when a small chemical molecule binds to large receptor protein, both molecules end up changing their shape. The fact that you get this induced fit in the complex may be sufficient to produce enough perturbation in a bilayer in a system.

We are beginning to understand what the receptors look like and how they function. For example, in the G protein-coupled systems, upon binding of the ligand there is a tremendous conformational change which makes potential sites of phosphorylation accessible to the kinase, which is a ligand-dependent phosphorylation. It doesn't happen in the absence of a ligand. We don't know all the mechanisms. Maybe there is a protein that is sitting on it that jumps off when it binds, but it's possible also that in arranging the seven times membrane domains by putting the ligand in, you effect a conformational change. Perhaps you can detect that other than worrying about coupling to the G protein in the cyclase. Maybe a particular antibody will recognize the activated receptor state and then it can be amplified. We need to understand much more of these coupling mechanisms and the real molecular basis.

Q: You could use an optical method for acetylcholine and get an optical signal. This is a case where you don't need the transmembrane, and protein could be a basal anion with collagen tail, by cutting up the muscle.

Eldefrawi: But that's just a case where binding of this particular ligand, thiocholine iodide, would give you an optical signal.

Q: The fact that there are receptors which are quite similar in structure but which have quite dissimilar function, such as the beta adrenergic receptor and muscarinic receptor, is somewhat reminiscent of the story with the immunoglobulin supergene family. Do you think that there are lessons here to be learned in trying to fish out the genes for receptors for which the biochemical information is very scanty in the literature? Examples would be catecholamine receptors, opiate receptors, and such things like that.

Ramachandran: Several people are trying to use that approach. The problem is if this serpentine membrane domain is so common, every time you go fishing you come out with too many clones. But if you have a tissue which seems to express uniquely a particular receptor or dopamine or something, then a cDNA library can be used with the general probe. If you can have a functional assay, then you can be sure that you are going after the right molecule.

Valdes: Yesterday was the life sciences section. We talked about receptors and their complexes and proteins, and how to clone them. Today, we'll talk about the hardware and microsensors. The two topics which we'll discuss today deal with the interface between the receptors and microsensors and transducing signals obtained once the receptor is placed on the thin film polymer solid support, and then we'll talk about microsensors. To get the hardware people who work with the surface chemistry and the various microsensors into thinking about the issues, I'll give a brief overview of the basic terminology of receptors and their characteristics.

A receptor is a large protein which, when occupied by a transmitter, will modify a cell. We want to mimic the function of the receptor or the function of a cell. There are many different receptors and receptor subtypes, and they're pharmacologically distinct. This raises further issues; we can talk about a cholinergic receptor but then we have to break it down into various subtypes, and we have to decide which ones we are interested in putting on the microsensors and what special characteristics each subtype has. These are classically defined in terms of what compounds, what agonists or antagonists, they will bind. A receptor-based biosensor would be expected to detect the whole gamut; hormones, growth factors, toxins, opioids, various peptides, down to the classic transmitters like catecholamine, serotonin and histamine. These molecules are informational molecules that are of physiological relevance to the detection of environmental contaminants, whether it's on a battlefield or in a toxic waste dump, and for diagnosing disease states. The receptors themselves are relatively unstable.

We've identified two major areas where added research effort is required. One is in the production of the receptors. Yesterday, you heard at length many elegant studies of cloning genes to express receptors, and so the potential for producing them in relatively large quantities exists. The other major technological bottleneck is going to be to immobilize and stabilize receptors on some sort of a solid surface that can be coupled with the microsensor. We can also classify receptors in terms of what happens after they bind a ligand. The transmitter will bind at the recognition site of the receptor, its active site, and then the G protein activates the conversion of ATP to

cyclic AMP. Our challenge is going to be to mimic the physiologic aspects of this lipid bilayer and yet have it stable, and maintain it as a stable solid support so that the receptor is essentially fooled into thinking that it's sitting in its native environment. The other group is receptors that are coupled to a channel. If the receptor is active, there's a gradient of ions across the membrane and the channel is closed. When the ligand binds at the active site, it opens the channel and ions flow through. The importance is that the design of a receptor-based biosensor should take into account different transduction strategies for different classes of receptors. A channel coupled with the GABA receptor has various sites on it for drugs and toxins which will open or close its chloride channel. An acetylcholine receptor sits in the bilayer, and has five subunits, alpha, alpha, delta, gamma, and beta. Typically, when we are studying receptor function, we can do so with various approaches in the test tube with binding assays or with a patch clamp. A patch clamp is a microelectrode which has a lipid bilayer which is seeded; it forms a bilayer with the receptor in the bilayer. From the pharmacologist's point of view, this is a very elegant and powerful tool for studying receptor function and drug and toxin interaction with a receptor, what happens once they do, and the transduction of signals. But it's a very fragile system which is not going to be useful as a true biosensor.

This is the challenge to the pharmacologist and biochemist: to take the principles which we learn from pharmacology, biochemistry and electrophysiology, and to then modify receptors to function in a true biosensor. I should say that's more of a challenge to the people who are surface chemists and people who are designing these biosensors themselves. Dr. Jess Patton, who is today's chairman, will introduce various techniques, approaches, and logical thought processes for putting receptors onto these solid supports and then coupling them to microsensors. Following his comments, we will discuss the actual microsensor technologies that are presently being studied.

Patton: I think there are a lot of issues concerning the design of sensors and especially how you're going to mate all this wonderful chemistry and biology to sensors and do it in an engineering manner. It's quite a challenge. I thought I would start out for our engineering friends, and perhaps others, by just doing a simple overview of biosensors. What is a biosensor? Basically, it consists of some reactant or analyte. The reactants will, in combination with the analytes, join on a bioreactor where recognition molecules are located and some selection process takes place. The selection process may also be accompanied by a change in chemical structure. That change is detected by the transducer. Those are the basic elements of a sensor. There are several varieties including optical, electrochemical and piezoelectric. We also want to take a look at some of the other transducer types and think about how they could be adapted to be used with receptors. I think receptors are probably where antibodies were fifteen or twenty years ago. There are other types of specific "receptors", including enzymes which recognize organophosphorous compounds, etc. DNA probes are a big upcoming field, plus the membrane receptors which Dr. Valdes discussed. You have to purify them, characterize them, modify them and perhaps simplify them. You may only want the recognition site within a molecule. Often with antibodies

that's done; the FAB fragments are sized and used. That raises a question: do we only need the recognition sites to make sensors, or do we need the whole molecule to perform a sensing function for us? Those, then, have to be stabilized on some solid surface. The most prevalent way is to covalently bond them. Microencapsulation is another technique. Other methods are crosslinking receptors and the addition of cofactors. You also need a stable, well-characterized, receptor site, and some type of signal that you can detect. High sensitivity usually entails using a tag such as redox tags if you're using electrochemical sensors. Optical sensors need things like fluorescers, luminescers or even colorimetric tags. When you have these interactions or competition between the analyte and the labelled analyte, you can measure the difference in the amount of analytes in a sample. They're going to compete for the binding sites, and the tags are what you detect. Volatile release tags are a biochemical reaction that takes place after a binding event. This generates a volatile gas that can be detected by mass spectrometry and ion mobility spectroscopy. You take those type of biomaterials and design an array of optical sensors, electrochemical, piezoelectric, and a specialized class of IMS.

I thought we might just take a very quick look at what some of the reactants are that one needs in making a biosensor. Here are some of the types that can be used: radioactive tags, such as those used in RIA in clinical diagnostics. Mass tags can be used in conjunction with piezoelectric or mass balance type of sensors, or surface acoustic wave devices. What type of chemistries can take place so that you end up getting selectivity? I'll talk about three. The first scheme called displacement, or hit and run. This has been demonstrated for T-2 toxin. One can detect one nanogram per assay range. One immobilizes the analyte of interest, T-2, onto a solid state surface and then pre-loads that with T-2 antibody, or to the analyte that's been labelled with an optical or electrochemical label. Introducing the sample results in competition for the antibody. The antibodies are actually in some kind of an equilibrium; there's an on-off mechanism going on to which some of the labelled antibodies move out into free solution. Nevertheless, we view it simplistically as displacement where one of these analytes can displace the tag and be detected by the sensor. You've got all the immunoreagents on one solid surface which has some advantages if you're looking at field portable units to be used either for military applications or for other environmental monitoring, air or water. The displacement scheme appears to work well for small analytes. It just hasn't been investigated enough to know if it's going to work very well for very large analytes. The second scheme, most widely used in clinical diagnostics, is the so-called sandwich reaction scheme in which you have sample and labelled antibodies, and on some solid phase or the reactor column, you have immobilized antibody. You introduce these and competition for these antibody sites results in a configuration in which the analyte, typically a large one, is sandwiched between the two antibodies, with some of the label remaining on the column. The amount of label that remains is proportional to the amount of sample. A lower signal means that you had more sample. That's a fast immunoassay compared to displacement. We heard some questions yesterday about the regeneration of the sensor. There's been work in that area. Alice

Wilson, in 1984, published an interesting article in which she demonstrated a sandwich assay with a closed loop system; by changing the pH and the buffer ionic strength, the column is regenerated. That was about a 20 minute cycle. It could probably be shortened up even more. There is some evidence that you can regenerate many of these immunoassays. The two previous assays I talked about are referred to as heterogeneous, meaning that one of the immunoreactants, either the antibody or the analyte, is immobilized on a solid phase. The rest of the reactant is in solution. The third scheme is based on having homogeneous solutions. A labelled antibody reacts with some of the sample antigen; the antibodies are tumbling, rotating, in solution at some rate. If the sample itself is a large molecule the rotational rate changes. Using optical labels, the change in rotational rate can be detected. That's known as fluorescence polarization.

Can you stabilize the molecules by covalently bonding them to solid surfaces? The answer is certainly yes. Is it going to help receptors? Probably. Immobilizing on the solid surfaces often will stabilize the molecule. Different types of coupling agents, different spacers and different chemical structures have been used. We did a study a few years ago in which we developed a technique to vary the bond lengths one molecular layer at a time, but not change the chemistry so that we could study in a more controlled way the effect of bond length on stabilizing acetylcholinesterase. There is an increase in stability with the increase in length. Silicon oxide surfaces with a salinization reaction gave us an amino group with a reactive linkage. An excess of a coupling agent, known as CDI, produced an intermediary which we reacted with a diamine and generated a linkage. In doing so we generated another amino group. That cycle could be repeated to control the length at the molecular level, keep the chemistry of the whole linkages of different lengths, and immobilize the enzyme to it. Using Maryfield's technique for taking amino acids and building up peptides and proteins, we've done essentially the same thing, but with synthetic reactants. We had radiolabelled phenylene diamine and measured the increase in activity per reaction cycle. After immobilizing the enzyme, we looked at the stability of each one of those linkages and found very fine control on the lengths, and the chemistries were the same. If you plot the percent change in bond length for each subsequent cycle against the percent activity, you get a very good correlation. This increased the stability of acetylcholinesterase by 120 percent over anything we had seen reported before. We're going to have to do similar things with receptors, and do them in a controlled way. Transducer technology is in its infancy. We're going to hear some exciting results today by some of our sensor speakers. There are several transducer types that we ought to be looking at: optical types, evanescent waveguides, microcells or optrodes, fluorotometry, plasma resonance, immunoparticle glutination, light scattering based and electrochemical types. Some involve measuring changes in current. Reactions take place on the surface of a chemfet; the charge changes, the current output of the chemfet changes. Dr. Cheung will talk about that. Arnold Newman will talk about some of the exciting results with capacitive sensors. I'll show you an example of a change in the transmembrane potential due to a binding event.

Many things can be miniaturized. Piezoelectric sensors are available, as well as surface acoustic wave guides. Depending on the mass that's on either a surface acoustic wave or piezoelectric crystal, the standing wave that is generated across the surface of that device will change in frequencies proportional to change in mass. When enzymes react, they turn over molecules at very high rate. That's a chemical reaction that generates heat which can be measured with sensitivities in the neighborhood of a tenth of a picogram or picomole. Is there enough heat reaction generated in receptors to take advantage of that? Some of the optical sensors are really exciting, and you'll hear two good papers today by Drs. Andrade and Block. Those are evanescent wave based; there are other types in which you simply use the optical fiber to interrogate, or transmit, the signal from a minireactor vessel which can be microliter sized. There are simple enzyme electrodes and the intent is to show you how you can take a classical electrode and, with a few simple materials, make an enzyme specific sensor. I mentioned chemfets. For those of us who are in the sensors business, chemfets are exciting; they've been around a while and we have an expert with us today who will talk about that, Dr. Peter Cheung, from the University of Washington.

Block: In reviewing and trying to present some scheme for this almost infinite number of sensor types, there's a drawback from trying to be too rational. Let me tell you a story and then I'll make my point. A number of years ago, there was a young man who had a business opportunity to set up a company to provide boards at airports so that you could determine when your flight was, what gate it was, and that sort of thing. I was supposed to be advising investors on this. He made a presentation and explained very carefully that even though it was possible to use a television screen, that would never happen because there were more bits of information available on a television screen than you actually needed. What happened in that time was that television became mass produced and very cheap. Even though it's tremendous overkill, we use a television screen. That's the way it went. In the sensor business you may have a parallel. There is a tremendous commercial thrust for biosensors. Regardless of rational reasons and rational choices I think the odds are that history will repeat itself and the sensors that will find themselves in the doctor's office, mass produced, made for pennies, and sold for dollars.

Q: You alluded to how important the format of immunoassays may be to working with receptors. What kind of data do we have to suggest that a competitive displacement format would give a sufficiently generic response in a biosensor using receptors?

Patton: I am not aware that data have been gathered in a very systematic way. Some of the early efforts tried to use mobilized or tethered ligands for competitive displacement.

Valdes: Dr. Chambers has been using the calcium magnesium ATPase calcium channel complex on the capacitive sensor, and it's a similar transduction scheme.

Q: You alluded to concerns with binding constant, displacement, regeneration and so forth, but you didn't say anything about nonspecific binding.

Patton: With these immunoassays, nonspecific binding can be troublesome. Concerning the nonspecific binding where the analyte comes in and binds not with the antibody but with some active electrostatic site on the surface, people are using protein solutions and surfactants and solutions of this sort to get rid of non-specific binding.

Q: I have a question concerning the immobilized enzyme. Were you measuring the corrected activity of the enzyme based on protein concentration?

Patton: It was acetylcholinesterase which catalyzes the hydrolysis of acetylcholine. We were using a simulant, DFP, diisopropylfluorophosphate, and measuring the rate at which it will catalyze hydrolysis. We measured the catalytic activity of the enzyme to a standard DFP solution.

Q: Was that amount of enzyme per the amount of activity; was it units of activity?

Patton: It was units. What I showed you was relative change in activity, and the 100 percent was the catalytic activity of that preparation at day zero or day one.

Q: Do you have data concerning the stability of the link itself, or were you were losing protein from your nodes or losing enzyme molecules?

Patton: I don't think so, because we prepared them covalently bonded, and it doesn't wash off. When you're doing a reaction you'll have some that doesn't react; you typically use an excess of enzymes when you do the immobilization. But that all gets washed very well with buffer so that we have a steady baseline activity with which to begin. So I think we compensated for that.

Let's get on to some of the more exciting talks. Mr. Arnold Newman, formerly of APL, and now with Biotronics as Vice President of Research and Engineering, is going to discuss capacitance sensors.

Newman: I want to start off by telling you how I got interested in sensors. My wife is an endocrinologist at the National Institute of Health. Several years ago she was doing a protocol where they wanted to measure the changes in hormone LH in a woman over a 24 hour period. Apparently, every ten minutes of that 24 hour period they drew blood. In endocrinology one of the hot subjects is pulsatility of hormones. There are certain hormones such as LH that will have very large spikes of concentration that last perhaps a minute. My background had been primarily as an electrical engineer and I started to look into the problem of sensors. I was rather naive at the time. I was amazed that there wasn't something you could stick in somebody's vein and measure hormone levels as they changed over time. Sometimes when you start out

with a rather naive and innocent approach to things, you may see things in a relatively simplistic, but perhaps less prejudiced way. From that beginning, several ideas came up and several of us had the opportunity to investigate them. At the present time, we're actively pursuing sensor development in various areas and finding corporate stability in more traditional works such as biotelemetry, microcomputer base systems, and process control systems, as well as some product development in purely traditional biotechnology areas. Our great interest is in the biosensor area, and given the difficulty of working with biosensors and developing them, it's nice to have the stability that accrues from this broad background. One of the things that an engineer does when he or she approaches a problem is to try to think in terms of the systems and how the systems interact with the environment. I would like to start off with a bit of historical perspective that motivates all the work that we're doing.

Throughout history, mankind had been administering chemicals to the environment for various purposes. The first gas attack by the Germans was on April 22, 1915. From the soldiers' perspective, this is noxious and obviously very dangerous. Protection was not always very effective, despite some very valiant efforts. Chemical warfare during World War I was quite a significant aspect of the casualty percentage with 31 percent of the casualties being related to gas. In World War II, Walt Disney was enlisted to design a protective mask for children. I would say that it is quite unfortunate that in some instances our chemical defense has not really progressed beyond this point. The use of chemical weapons by the Iraqis has been documented during the Persian Gulf war. There have been pictures of mustard gas leaking from unexploded bombs, and there is some rumor that there are other kinds of neurotoxins that have been used as well. Let's give a bit of background to the scope of chemical warfare agents. We have various kinds of substances; nerve agents, general poisons and toxins are lethal. Incapacitating agents which can also in certain instances be lethal are blistering agents, tear gases and psychometric agents. Herbicides turned out to have effects that are quite lasting, and are in some ways weapons without our knowing it. Then there are industrial chemicals that, during World War I, were in fact considered chemical weapons and used as such. At this point, many of them we would not even consider; we use them consistently in industrial processes, particularly in the semiconductor industry.

What I want to discuss is, first, a generalized concept of how we look at biosensors. Transducers include two types of devices, the sensors as well as actuators. In considering any transducer, we have to realize that there are two interfaces that matter. There's the interface with the environment on one side of the transducer, and there's the interface with the instrumentation that tells the transducer what to do. These two interfaces are absolutely critical. A typical biosensor starts with the interface to the environment. That environment is essential to understanding how you're going to go about doing your research and your development work. In non-military uses it could be a doctor's office, but that's certainly a more benevolent environment than the battlefield. The transducer itself is a device that is able to transform energy from one form to another. Then you get to the second interface, depending on the transducer, and then you get to an electronics system that does amplification and storage and

processing of the data. Ultimately you get an information output. Each part of the generalized biosensor has its limitations, and you have to be impeccably honest about what those limitations are and try to compensate with the other aspects of the system with which you can work. For example, we know that there are many incredible things you can do with microcomputers. Therefore, problems of linearisation that might not be possible to solve at the first interface, may be taken care of later. When we started to look at biosensors a number of years ago, we considered these criteria. A sensor has to be sensitive enough to detect concentrations in situ in the range of interest. In situ, here, is on the battlefield. The range of interest is something that varies according to the tactics of your enemy, to the meteorological conditions, to the efficiency of the sample acquisition. The sensor has to respond according to what you can deliver to it. What you can deliver to it is a problem that has to be solved as well. Furthermore, sensor specificity should be such as to eliminate interference; non-specific adsorption, temperature, pH changes. Sensor reliability is also a critical thing. One thing that is not necessarily remembered but is important to stress is the manufacturability of your transducer. The sensors that will be made are going to be the ones that will be manufactured using relatively established practices. Those aspects of the manufacture that are not well established should be relatively straightforward, controllable, and simple. Finally, for the particular application, you'll find that they probably will be relatively small sensors that are going to be carried by a soldier; you don't want them to intrude upon the mission.

With the particular sensor that I'm discussing today, we have concentrated on antibodies as our receptors. The second aspect to making our sensor is to use the microelectronic fabrication techniques. They are relatively well established; it is easy to batch them up to great quantity production, and you can do upwards of one hundred wafers at any particular time. I think the cost to the manufacturer in terms of pennies is quite a reasonable expectation.

Transduction is the absolute essence of a sensor; it's a process by which a chemical event is converted to an electronic signal. It's the essence of the sensor, but also its weakest link. Therefore, in designing a sensor we should try to choose a transducible, physical, chemical property that one would find in substances that are generic. This particular type of thinking should relate to properties that function as the concentration of the substance functions. We chose to look at capacitance. Capacitance can be electronically measured with great precision. It's a function of several parameters, one of which is the dielectric constant, which is an inherent physical and chemical property of any substance. As immobilized antigens interact with antibodies in an aqueous environment, the dielectric properties near the surface upon which they're immobilized change. We've configured a sensor that exploits this antigen-antibody model, and because the interaction is specific, it has a potentially high signal-to-noise ratio. From the electronic point of view, and from the second interface I discussed, it's a measurable quantity; we measured precisely with a limited amount of electronics. As such, we have a relatively simple measurement to make. Our prototype sensor is adaptable to microelectronic circuit fabrication technique. This satisfies the manufacturability and size parameters I discussed. What

we have is an immobilized analyte, or hapten, on the surface of the outer layer. That analyte is covalently bound to the surface, and antibodies are allowed to bond or conjugate with the immobilized hapten on the surface. We provide an alternating electric field between the opposing metallic traces with the majority of the field being kept relatively close to the surface. When free analyte, which is analogous to the immobilized analyte or hapten, comes through the molecular sieve membrane it will displace some of the antibody in proportion to the concentration of free analyte. It will pull some of the antibody out of the field. When the free analyte concentration in the environment decreases, mass action laws are such that a free analyte will wash through your membrane and the antibody will go back onto the surface. The critical thing here is choosing the correct molecular cutoff size of the membrane. The antibodies that we're talking about are about a hundred and fifty thousand daltons in size. The sensor is made up of two or more planar capacitors. One of these capacitors is configured with the analyte and analyte-specific antibody of interest. We also have another capacitor that's part of the system which serves as a reference capacitor. Immobilized on its surface is what we call a dummy analyte which is a molecule with no affinity for the antibody of the real analyte, an antibody of the same class as the real analyte-sensitive antibody. You choose an analyte that you would not expect to see in the environment. The reference capacitor behaves in the same way to the nonspecific effects that one would see in the environment: temperature effects, pH effects, ionic effects, and nonspecific adsorption effects. Essentially, you're starting off with a sensor that is comprised of two capacitors, one the reference and the other the test. It may be that you know your environment quite well and, therefore, don't have to worry about certain nonspecific effects because you're controlling them. Then you might keep your reference capacitor simple.

The antibodies sit on the surface of these molecules that are presenting immunologically from the surface. When a free analyte comes in, appropriate molecular engineering has allowed the antibody to be displaced. The initial interest in T-2 occurred in relation to reports coming out of Southeast Asia about its use. The T-2 antibody is relatively specific. Surface chemistry is that first interface. We first silanized the surface by adding a gamma amino propyl triepoxy silane to the device bathed in the appropriate environment and found that you can bond this gamma amino system to a silanol on the surface. This allows us to have a gamma amino group on the surface similar to what Dr. Patton discussed earlier. The next thing you want to do is to succinylate the T-2 toxin to come up with a hemi-succinate T-2 and this is done by putting T-2 in the presence of pyridine and heating it with succinic anhydride. Once you have this, you can take the T-2 hemi-succinate and with the gamma amino function of the silanized device in the presence of water, bond it with an appropriate soluble carbodiimide. We used a one-sided system with no reference capacitor at that time. We measured the capacitance with a 1657 digibridge. The T-2 antibody we used was added at 1 microgram per ml concentration. That particular affinity constant was 5.8×10^7 liter moles. It's interesting because in any system like this there are many parameters with which you can work. These are things like the parameters relating to the receptors, such as the affinity constants,

and whether you want to chop up the molecule and use certain aspects of it. We added the antibody and, over a short period of time we saw a drop from 2180 down to 2120. When we added T-2 we were able to displace the antibody off the surface and get a return of the measured capacitance of the original value. The ultimate idea is to come up with sensors in certain configurations that would be packaged simply and plugged into a cable and used. The application defines the packaging of the sensor, and the sensors to be used in the battlefield will be packaged quite differently from the sensors to be used in other areas such as in the environment, in the doctor's office, in the hospital, and in process control applications.

Recently we've been continuing development of our sensor technology using smaller sensors and a different configuration. Pentachlorophenol is a pollutant found around paper plants and in the lumber industry, and also used as a preservative in wood. In an experiment where we have taken the antibody and added it to a system, you see a change in capacitance as different concentrations of the antibody are added. You'll see that the changes that we're talking about are less than 300 picofarads. To give a sense of what picofarads are, you can go to Radio Shack and buy a 3 picofarad capacitor. It's quite possible to resolve down to tenths and hundredths of a picofarad with straightforward and simple electronic instrumentation. A 250 to 300 picofarad change for a dynamic range in this particular system is a perfectly healthy system. What we've also done is added anti-T-2 antibody, which is nonreactive with the immobilized pentachlorophenol antibody, and preinhibited it with pentachlorophenol at a 1 to 1.5 ratio of the antibody to the PCP. Nothing happens. We're not exactly sure why this occurred, but one of the things that we surmise is that the antibody is a divalent antibody; it has two binding sites on it. We probably should have used a 1 to 2 or even greater ratio in the inhibition process. This is a relatively changing capacitance in parts per thousand, and antibody is added in nanograms. This was not a monoclonal so we added in terms of grams instead of molarity. We get a continual decrease associated with concentration or total antibody mass added. Then, with the same system, we did a different experiment. We had used a monoclonal, and it was saturating the surface. We pulled it off by adding free cortisol hydrocortisone, giving a sharp rise which saturates. Our first commercializable product is a sensor that's analogous in function, but not a biosensor per se, for medium chain aliphatic hydrocarbons. It has applications in the petrochemical industry in terms of process control and exploration purposes. We're able to sense hydrocarbons of medium chain aliphatic hydrocarbons in water as opposed to in the gaseous phase. That becomes a useful thing to do if you're looking at ground water or if you want to control a petrochemical process. In fact, some of these things will eventually lead to commercializable, manufacturable products and they will also find their way into the military arena. The kinds of packages that you use in the military arena, the packaging and the delivery systems of samples will be different. Conceptually, you're going to find that there's going to be a certain amount of overlap between the different uses which will feed each other.

Q: Do you have any sensitivity data for the immunoassay?

Newman: We have had experience in the picomolar range, and found that you have to configure your system for the environment. In our development, we have found that there are many ways in which we can place the sensitivity curve in the window of interest. By manipulating certain parameters, we can make it more or less sensitive. Sensitivity is use dependent. For example, with our hydrocarbon sensing system, the interest is in one ppm to fifty ppm even though I can measure down to one ppb. The immunoassay can easily get down to the picomolar range.

Q: Do you have any data on binding curves; what happens as a function of time when you introduce analytes?

Newman: The binding curves are reasonably stable and competitive with EIA type techniques.

Eldefrawi: Would you be kind enough to elaborate a little bit about the kind of molecular sieve you've used; number two, how essential is it really to get the measurements we're getting?

Newman: We have not been concentrating on the membrane development for several reasons; one, there are useful products without a membrane and two, there are other techniques that we can use for rejuvenation, for example, the chemistry of the sensor. Therefore, even without a membrane it's a useful sensor. I might say also that what I've shown today is a competitive system; in fact, if we have large molecules that happen to be the analyte that we're looking for, I can put a binding machinery on the surface and bind those large molecules and see them. I can see a picomolar of a large molecule; I cannot see a picomolar of a small molecule. That's fine, because then I just reverse and use a large molecule as the signal generating element. For example, if I'm looking for antibodies in serum, then I don't necessarily immobilize another antibody on the surface. I might immobilize an antigen on the surface. The presence of those antibodies in the serum could be sensed. The membrane development is not a minor task; it's an established technology.

Q: Could you comment on the use of silicon oxide over silicon nitride; that seems to be an extraordinary choice of passivating layers.

Newman: That was very early work and the idea was to try to find a system which was easy to bind to because there was more silanol groups, less stoichiometric mix. We have found, in fact, that silicon dioxide is just fine.

Q: Do you find that there's electromigration or drift in the silicon dioxide?

Newman: These sensors may take a certain amount of time to stabilize, but once they're stabilized you find that there's no change because that outer oxide level is so thin, it's only used for binding purposes; not for the passivation of the nitride.

Q: You don't find enough hydrated oxynitride on the silicon nitride itself to bind?

Newman: We also have used an oxynitride, and that worked pretty well. One of the things that you must consider is the process as a whole. If you want to keep your process cheap, sometimes it's better to use aluminum metallization and do relatively low temperature types of depositions. You don't have the complete freedom to choose any passivation technique you want. The best nitrides are the thermal nitrides done up to a thousand degrees, but that requires that you use a relatively fancy refractory metal in your metallization or some other kind of conductor.

Q: As I understand your T-2 sensor, it's a true sensor in that it will respond to fluctuating levels of T-2 in the environment. That means that your antibody is being contained, presumably, in a small volume held between the sensor surface and the membrane, thereby being reused continuously. What is your experience with lifetime of such a system; what is your experience with long-term adsorption and denaturation effects of the antibody on the silicon nitride or silicon monoxide, the untreated parts, your experience with non-specific binding, your experience with antibody interactions with the inner surface of the membrane itself?

Newman: We have not really been concerning ourselves with long-term stability of the biochemistry beyond one or two months. You're going to find that the kinds of technologies that are already using antibodies and that are being sold will presage the lifetime of your antibodies. For example, the shelf life of an HIV test, the EIA, is somewhere around two months. This is a true market tested system and the biology is a two-month biology. The benefit of working with our hydrocarbon system is that it also has a reference capacitor in it. We can have temperature changes over a large range and, by doing clever signal processing, differentiate those things out. We also have analogous kinds of happenings that occur in terms of non-specific binding, etc. If you configure your reference system appropriately, you can see those effects happening to both sensors and, therefore, differentiate those effects out.

Hallowell: I appreciate your statement that somebody's successful experiment today is your engineering nightmare tomorrow. I see that you envision your sensor as a reversible sensor because of the membrane. Is that the way you're using it?

Newman: That's not the way we're using it now. There are reasonable product uses where you don't use the membrane at all. Some of them are direct measurement or detection of antibodies themselves in a non-competitive form and others are rejuvenating systems as was mentioned earlier.

Hallowell: I was pleased to see the affinity constant of the T-2 antibody was about 10^7 and that's certainly comparable with the affinities you see with the receptor protein. I'm hesitant about people who talk about making direct analogies between the antibody-

antigen experiment where typically affinity constants are much higher. It's good to see that kind of sensitivity with a lower affinity constant.

Newman: Depending on how you make your antibodies, when you harvest them, and which ones you choose, you can come up with lower affinity constant antibodies. Reversibility is not a simplistic term. If you want a system that's reversible, you choose different parameters in different levels than if you want a non-reversible system. There are many applications for non-reversible systems, such as disposable sensors or detectors.

Hallowell: Do you envision making an analytical device or are you simply making a sensor? Let's propose a sensor that ultimately is at the bottom of this project. Do you envision a sensor which is developed in which a yes-no answer comes out as a result of perhaps 10 percent occupation of receptor sites? How do you think this is going to work?

Newman: I'd have to say that a lot of our decisions are market driven. The markets tell you what people need and then you think about what's simple enough to produce relatively quickly and start selling things. You will find that there are uses for all the kinds of configurations you mentioned. It's easier to make a yes-no device. People in Bhopal would have been very happy to have had one. There are many applications in process control and in the military where a continuous on-line sensor is very important.

Q: If I might make a historical comment, which has to do with the use of capacitive techniques in electric chemistry. The use of capacitive techniques in 1935 was a way of measuring trace amounts of material in solution. There's a large literature on this and a very large theoretical background on how capacitive changes occur when you have a conducting electrode in contact with solution. There has been development of this in terms of having insulating films on electrodes. This is a fairly well developed area. Until this sort of work that was done, it was generally not an area that people liked to work in because it was non-selective. It's the idea of binding things to the electrode to give selectivity that couples to the tremendous sensitivity that is available this way. But it's really not an area that one can't predict a priori and the sorts of sensitivities one can obtain. This theory has been worked over by people who spent their life at this sort of thing, and studied double-layer theory.

Newman: Absolutely. But I would mention that we are outside of the double-layer. In fact, that was one of the things we wanted to avoid as our signal because most double-layer work has been done with mercury and cleanliness on the metal electrode is essential.

Q: I think you missed my point. The capacitive effects you are seeing are the result of series capacitors and the final thing that's responding is a species that sits on an insulator and the way you couple into solution is through the electric double-layer. The changes in the surface adsorption and configuration affect the material that's

sitting on the solenoid surface. Ultimately, the description of the process will involve the description of the compact double-layer. I presume you're working on fairly concentrated solution, and that changes when you bring in a specific substance. That is the analytically sensitive area. The changes are occurring at the interface on both of those interdigitated electrodes. The changes you observe will depend on the absolute potential of those interfaces with respect to a reference. It is not a bulk phenomenon in solution; it is an interfacial phenomenon. There are electric fields there. That solution is a conductor; really my comment is a critical one on that one diagram in which you showed the field lines stretching from one electrode to another. When you add the solution to that area, that solution is an electric conductor so the entire potential has dropped across your insulator and across the electric double-layer. Everything you're seeing is changes in the capacitance of the double-layer. I think you need to look into this literature which was just talked about over the past 20 years looking at the electric double-layer.

Newman: The system is not as simple as one would think from the perspective of the double-layer. I agree that there is conduction through the medium but I can't really go any further than that.

Q: Earlier, competitive immunoassays are usually optimal in the limit of zero concentration, so that would suggest going down to very small device sizes. What sets your limits? You're talking about picomolar; is it how large a capacitance you need to have or is it steady state, or denaturation type losses of very small amounts of materials?

Newman: We used EIAs as part of our validation protocols. If we get a sample of, for example, serum, we'll run it on EIA and compare the sensitivity of the system to the capacitance device. We are able to configure these things by playing with various physical and chemical parameters to be in the sensitivity range of interest.

Patton: Our next speaker is Professor Peter Cheung, who is director of the Microelectronic Laboratory, Washington Technology Center, at the University of Washington in Seattle. Prior to that, Pete was Professor and director of a similar laboratory, Case Western, and has worked on microsolid state chemfet devices as well as optical sensors for many years. It's with pleasure that we welcome you, Pete.

Cheung: It's my pleasure to be here. I am a relatively naive person in the receptor-based sensor business. On the other hand, we have begun to think about doing some microsensor work in this area. I thought that this would be an excellent opportunity for me to throw some ideas out and have some interaction and certainly welcome comments from the experts among the audience to see if I'm even close to the right path. The title of today's talk is "From Chemfets to Reactor-Based Biosensors", and I would like to go through a bit of historical perspective of the chemfets. First, I'd like to bid you welcome from the Washington Technology Center; it's a newly established center at the state of Washington as an industrial university initiative. It has been functioning only for the past two or three years. Within the Washington Technology Center, we have a microsensor research program

which I initiated only about a year and a half ago when I relocated there. The microsensor research program is primarily dealing with applying microelectronics technology to fabricating sensors. Earlier, we heard about the advantages of sensors, using microelectronics technology in the real world for either medical or agricultural applications. In the military applications and in the chemical warfare detectors, you need to be able to manufacture them at a low cost. There are some other advantages of microsensors. We know about ultimate costs and its small size. One of the most exciting points in the upcoming future in microsensors is on-chip signal processing. We need to be able to couple the signal into an electronic scheme so that we can derive out the signal. In many instances, if we can combine the microelectronic technologies with biosensing capability, we could do complicated or complex signal processing schemes on the same chip. Eventually it would be possible for the sensor to have solar cells on it. It could be cell powered, have telemetry circuits, and could collect information without actually having the limitation of running, so it can be scanner run in a wide field area. These are some of the potential possibilities. In the Washington Technology Center, the microsensor research program is interacting with a number of other research programs. Primarily we are interested in using sensors for advanced manufacturing for medical applications and in biotechnology.

I would like to turn my attention now to chemfets, and from chemfets to bioreceptor sensors. One aspect of the chemfets from the very early days is the ion sensitive field effect transistors. They are not much different than a regular MOS field effect transistor stemming from the metal oxide semiconductor field effect transistor. The principle of the metal oxide semiconductor field effect transistor is that you have a capacitor mechanism which is separated by the metal and insulator, and the semiconductor as the lower plate of a double-layer capacitor. By applying a potential on this capacitor, we can control the current flowing between two electrodes on the bottom plate of the capacitor. These two electrodes can be externally biased, and you can realize the effect of amplification of the input signal. Another advantage of the MOS FET is that it is a capacitor; there is tremendously high input impedance for isolating the potential you want measured with respect to the output of the device. This is important for the case of a sensor or electrode where you do not necessarily want to disturb the potential that is generated by the medium, or the transduction mechanism. The FET had the advantage that, because of the very high isolating impedance, it will not disturb the phenomenon to be measured and, subsequently, lends itself to a more highly accurate capability. The only difference is that the solution sample we want to measure is now inserted in between the metal electrode which is represented by a reference electrode in the solution interface, and the insulator of the FET. Now we have two series capacitors connected in between the gates in which there is the insulator capacitance as well as the electrolytes and also the metal gate. We can also refer to the ion sensitive FET as a removed or remote gate FET device. Sometimes it's also called a bare gate device, where the insulator itself is used for sensing. There are modified devices where the insulators are chemically modified, and there are coated devices where the insulators are coated with a polymer which is responsible for the sensing. This is a basic principle of the FET. If you have the ion sensitive field

effect transistor, the sample solution, and the reference electrode which represents a remote gate, then you can bias it. Any electrochemical potential that can form in between the reference electrode and the block gate insulator can now be used to modulate the current between the train and the electrode, and subsequently generate a current.

Starting with a silicon wafer, you can do oxide masking and train diffusion, which is essentially the second mask. After the train diffusion, we form the oxide gate and open the window from the very pure layer of gate oxide anywhere between five hundred angstroms to one thousand angstroms. After the gate oxide is done, we usually carry out an additional step where silicon nitride or other types of inorganic oxides are deposited because the silicon dioxide is not necessarily a very good insulating layer for sensing. The fourth step is doing the metal contact with the train electrodes, and then passivation. Basically, it's a very simplified step which usually involves no more than four masking steps which in the semiconductor industry is a very rare tactic. In the sense that it is simple, it is also a discrete device which gives very high yields. For example, in a three or four inch wafer, you can typically have yields from five thousand to ten thousand sensors on one wafer. We heard the earlier speaker mention that you can build fifty to two hundred wafers on the lot and it would take about four or five days to build one lot of these wafers. We are trying to get higher transconductance by using an interdigitary gate channel configuration to increase the current gain.

Aluminum metal is used for supporting an MOS FET to support the ion sensing FET on the same chip. The dimension of this chip is slightly less than one millimeter to two and a half millimeters in size. If I'm going to build only a single FET, it can be built in this size or maybe even a tenth of that size. Even with one by two millimeter size you can still have a yield of several thousand on a four to five inch wafer. Typically, the yield of this device today for discrete semiconductor industries is 90% and above.

There is some mystery to the theory of the ion sensitive field effect transistor and I would like to briefly explain how it operates. I have added a work electrolyte because it is an intimate component. The sample component we are measuring is an intimate component of the overall measurement system. In a simplified manner I have just discussed the train electrode only to provide the active region of the transistor. The transduction mechanism is based on the electrolyte sample we want measured and the insulator. In this case the insulator may be silicon dioxide. It's the electrolyte insulator semiconductor structure that is ultimately responsible for the chemical response. When silicon dioxide is in a wet condition it surface hydrolyzes into positive sites and negative sites. This association-dissociation formed the tos centers on the surfaces and there is a controversy in terms of the discussion in the capacitor sensor in the last presentation. The electrolyte and the potential are certainly related to the semiconductor insulator and the semiconductor interface through the compact ionic layer. Through the upper layer the bulk potential can be related into the semiconductor potential profile. We can state the physical principle of the mechanism, but we need to be able to quantify the chart potential distribution or the chart's density. First, we need to know the association and dissociation

constants. The electrolyte has other ion species which can also form complexation, and the surface complexation can also modify the association-dissociation into SiOH groups and pick up protons at the surface charge and then associate and dissociate into this equation. You can characterize it based on a dissociation constant. You can also have silicon dioxide hydrating and dissociating into the negative charge site and surface hydrogen proton concentrations characterized by a negative equilibrium constant. The proton concentration determines at what pH the amount of surface charge of the SiOH group would merge into the positive and negative task site. For example, at a surface pH where the negative and positive sites are equal, we define that pH as the pH at the point of zero charge. So the surface charge density can be related to the function of the surface pH. The material characteristic equilibrium constant and the separation of these two constants is sometimes called the Delta pK. This is the log of the dissociation constant value that determines how much of the SiOH groups can be dissociated and associated into the plus or negative charges. The difference between these two values is the ability of SiOH to dissociate discharges and we can predict chemical response. Surface complexation is a means of supporting electrolytes. We use an example of sodium chloride as a supporting electrolyte that can also form complex charge centers with the SiO⁻ and SiOH²⁺ species. These also have association and dissociation constants and can modify these constants. With the distribution we can now relate the charges on the surface with the charges in the compact double layer with the part of the electrolyte. With the charges quantitatively described, we can relate this potential of the insulator or the semi-conductor. Subsequently, any change on a potential determining ion species or interference species, whether it is at the part or at the double layer, can be used to modulate the semi-conductor potential. If we know the semi-conductor potential across the insulator semi-conductor surface, we can use the available equations relating to potential or the field distribution and the current modulation theory. With this, we can completely characterize the ion sensitive field effect transistor in a quantitative manner that relates the electrolyte concentration, the concentration of the potential determining ions, and the output of the drain current or the gate voltage. This can be described in familiar field effect transistor equations which have the conventional electrical terms that describe the charges on the semi-conductor and the insulator, and also the chemical terms that relate to activity of the hydrogen ion. From this equation, the chemical information, and the dissociation constant, we can look at interference effect as well as chemical sensitivity effects. We would like to see if the theory is correct by doing some matching with experimental data. The theory predicts that with a very low supporting electrolyte, the pH changes, and with a very high supporting electrolyte the surface potential would change. The surface potential of the SiO₂ can be dramatically different as a function of the supporting electrolyte. All of this is not taking into account the interaction of the supporting electrolyte. The chemfet for pH did not follow the pH gas electrode theory. The Delta pK of the separation is between the association and the negative dissociation of the SiO₂ and SiOH, on the surfaces. The chemical sensitivity or the pH response will increase as the site density and chemical sensitivity increase. The site density predicted that the

chemical response would be on the order of about thirty to forty millivolt regions. If we can change the material according to the theory, and lower the Delta pK, we will have very high sensitivity close to the nerve's response. The higher the site density, the better the selectivity. The lower the Delta pK, the better selectivity of the surfaces. We can now search for materials that can offer either high surface site density or very low Delta pK. We can also search for where the pH of the POC lies. Researchers have looked at silicon nitride and aluminum oxide because aluminum oxide has a very low Delta pK value. Subsequently, even at site density of 10^{15} you will have very close response and improved selectivity. On a theoretical basis there would be SiO₂ surfaces in terms of pH response. There would be silicon nitride surfaces and aluminum oxide because of the much lower Delta PK. We are increasing the response due to the increase in the surface site density. With LPCVD aluminum oxide we can see that the response is close to electro response over extremely wide peaked ranges. This is one of the explanations of the electrolyte's semiconductor insulator transistor theories. We can now try to utilize the site binding theory to extend our work toward chemical sensitive FETs for biological agents and toxins. The first approach is an antibody-antigen system coupled to the FET. That system is highly specific because of the antibody-antigen, but has the disadvantage of not being a very generic sensor. We can also use enzyme-based sensors which can be more generic, and the bioreceptor sensor which, if it can be coupled onto the surfaces, would be the most generic type of sensor. Because a receptor-based chemfet would be the most generic type, we have chosen the receptor-based, or protein-based, biosensor. This approach can be applied to realize the potential of the chemical transduction so we can make a more general protein-based microbiosensor, or MBS.

The requirements for the protein-based MBS are still the three S's for sensors: sensitivity, specificity, and stability. Our basic principle is to use nature itself to do the sensing. We are going to appoint a very specific class of naturally occurring membrane proteins which nature uses for chemical detection. There are two types of proteins that can be found in the cell membranes. One is the insoluble proteins that are bound on the outer or the inner membrane of the cell. The second type is the soluble proteins that are usually dissolved in the periplasmic space of the cell membrane. These proteins serve a dual function. They are responsible for active transport of nutrients across the cell membranes, and can also function in chemotaxis response. There are also chemoreception proteins in the membrane. We have a petri dish in which are four mutant cells and in the center is a tiny drop of sugar substance. One mutant cell has a chemoreceptor protein that is sensitive to sugar, and is also responsible for the active transfer of the sugar across the membrane. The other three cells have no ring around them because they do not have the ribose receptor protein and do not respond to it. The popcorn receptor protein, which is responsible for the smell of popcorn, is one of the most sensitive receptor proteins in the nose. These receptor proteins can be isolated, identified, purified, and chemically characterized. These are crystal proteins that specifically bind to leucine, which we have isolated, purified and also crystallized. In order to apply this naturally occurring receptor protein to microbiosensors, we need the protein to have several characteristics.

It needs to have high specificity for what we want to sense, high affinity to trigger a high sensitivity signal, high stability so that under various conditions it will be stable for long term, and a very low sensitivity to pH, ionic strength, and temperature. A ribose bound protein has very high sensitivity in the nanomoles per milligram protein response and also it has an extremely broad pH optimum. This protein will work anywhere from pH3 to pH11 and is stable over these conditions. The same protein worked from water having no ions, all the way to 4M salt, and it's still functional. You can boil the protein for about 10 to 15 minutes, and it still would function. These kind of proteins would be very attractive to use as bioreceptor sensors. Here's where the engineering comes in. We would like a system approach where we could identify the receptor protein that we want to utilize; we need a way to purify and crystallize it, so that we can chemically characterize it. Once it is characterized, we can use it for the biosensor. The receptor protein must be able to respond to a labelled radioactive substrate or a toxin that is radioactively labelled. When you label the substrate or the toxin you can react it with the protein and then separate all the cell membrane proteins with high resolution, two dimensional, electrophoretic gel. After separation, you can identify, purify, and sequence it. It is now biochemically characterized for the coding DNA sequence which is responsible for the receptor mechanism. There is a protein identified as a receptor protein that binds to leucine and a couple other leucine-like amino acids. This protein would very specifically bind only to L-leucine and nothing else. There are two proteins, a mutant from one strain, and another receptor protein from another strain, that both bind to L-leucine. We must have an economical way to produce this protein through genetic cloning in bacteria in high concentration and high purity. Normally, these proteins are produced in the periplasmic space. We use a damaged cell wall technique where the proteins are genetically engineered, so that the receptor protein is secreted into the medium at high purity. If we can clone bacteria cells to produce the desirable cell protein for us, then the next thing to do is build a bioreactor. One cell shows all the different membrane proteins that are produced. Another is a genetically cloned type of bacteria cells, E. coli cells that produce a phosphate binding protein in large quantity. There is a cell that does nothing but actively produce only the phosphate binding proteins. The bioreactor would now require us to bind this membrane protein and immobilize it. We clone these cells which are suspended in a nongrowing condition, then immobilize them on the chips and put nutrients on them. They can produce the receptor protein in large quantity and in pure form into the medium. These cells can be thrust into a concentrator dialyzer where the waste is separated from the bioreactor product. There are bioreactors that have been run continuously producing these binding proteins over a one month period.

If we can produce these receptor proteins inexpensively, the next step is to utilize them for surface immobilization on silicon substrates. This involves two steps: the first step is to activate the surface, then attach the binding protein. The surface sites are very small and are not effective in hooking the binding protein. The next step is to do an intermediate chemical modification so that we can passivate the surface sites of the SiO_2 layer to be able to link

bigger hooks. From the bigger hooks we can use vapor phase deposition technique or reactive polymerization technique to build up these surfaces and attach bigger hooks onto it. The hooks are big enough to hook onto these receptor binding proteins. These proteins are very rugged; they can be created such that they operate in very wide pH ranges, wide ionic strengths, and even wide temperature variance. The surface is passivated so that it doesn't respond to other ions in solution. We have a bioreactive surface that can be used to couple into the silicon dioxide and silicon surfaces. These binding proteins are in the presence of the substrate; it will bind the substrate, forming a charge couple and will be detected electronically. The chemfet is an electronic amplifier, giving you a higher sensitivity and inherent gain as well as compatibility with microelectronics. You can easily link other microchip circuitry on top, forming a protein-based microbiosensor based on chemfet structures. We can also utilize other approaches in biotechnology and microelectronic technology for forming other microbiosensors. We can selectively do multiparameters because we can screen different types of binding proteins as well as substrates on the surface of the chips. Many of these proteins have another interesting property. Not only can they bind substances, they can also release the substance and be denatured and used again. For example, the phosphate binding protein can be immobilized on the gel and then it can, with phosphate, bind and then be released. Once it is released, it's denatured, but it can be renatured and then go through the whole cycle again. A cadmium ion binding protein will bind cadmium, release, bind, release; again, we can do this many times. Even after storing it dry in the laboratory over a month, it works again and again. We can use this for in vitro detoxification or separation applications.

In summary, we are proposing a systems approach to receptor-based microsensors using the aspect of the naturally occurring protein which is used for sensing or binding the substances as our sensing mechanism. We can separate, purify, and characterize it. We can also remove variables or add variables into the protein, because we only need is the DNA coding sequences that are responsible for the binding domain.

Q: Do you have any results from the analyte binding experiments? You showed the pH sensitivity, but nothing from the binding.

Cheung: We have some results on phosphate binding protein that worked in very broad pH range and ionic strength.

Eldefrawi: Would you elaborate on how the chemfet sensor translates binding of substrate to a signal? How would that differ if the protein bound any nonselective, nonspecific ligand, an ion for instance, or allosteric effector of some sort? How would this look, as a different signal or different effect?

Cheung: When the binding protein binds the substrate, there is a reorientation of the charges, a dipole reorientation or three dimensional configuration of the charges. This essentially couples through the compact double layer onto the silicon surfaces. It will have some sensitivity to other proteins on the bulk of the solution. But we are counting on a static case; we are talking about

electrostatic potential and so its sensitivity does not extend far enough into the bulk.

Andreou: Have you made the alumina insulating devices, and how did you make them?

Cheung: We have spent quite a few years making the aluminum oxide devices by means of a low pressure chemical vapor that uses LCV vapors.

Q: You draw a very sharp division between your silicon dioxide hydrates to a depth of several hundred angstroms. You now have a three phase system. Can you explain how that affects your model?

Cheung: Let me comment that there are different types of silicon dioxide. For example, glass does hydrate to a couple hundred angstroms. The thermal oxide is very tight, very compact and doesn't necessarily hydrate to that depth. The silicon dioxide is not a good material to use for pH sensing for chemfets; I would not recommend it since it has suboptimal response. If you are interested in pH, then use aluminum oxide or silicon nitride, which has very little hydration.

Patton: We will continue the afternoon session with Dr. Gary Rechnitz from the University of Delaware.

Rechnitz: Thank you very much and good afternoon ladies and gentlemen. Some of the work that I've heard described today is extremely interesting. In our laboratory we have had an interest in biosensors for many years, and only one of the subdirections of that overall effort has been devoted to receptor-based biosensors. I would like to take the time today to touch upon two or three possible strategies that could be employed for the development of biosensors. Biosensors are important to us because what we cannot measure, we cannot understand. Nowhere is this more strikingly true than in biotechnology and medicine. It is appropriate that there should be an intensive effort to find means of detecting, quantitating, and measuring biomolecules in various matrices. One can argue that many of the subareas in medicine and biotechnology are presently limited by our inability to make meaningful measurements of molecules in these fields.

My talk today is entitled "Molecular Recognition Elements for Biosensor Design." What I'm alluding to is the fact that biosensors generally consist of two components: a biological or biochemical component which is the molecular recognition element; and an electronic, electrochemical, optical or other instrumental component which provides the signal. As other speakers have already pointed out, the key to the development of biosensors lies in the coupling of the molecular recognition element to the instrumental component of the biosensor. This coupling is important because it determines the selectivity and sensitivity of the response. Since this conference is entitled "Receptor- Based Biosensors", I would like to begin with some recent work from our laboratory in which receptors are employed as molecular recognition elements. The first portion is one that is centered upon the need to amplify the binding event in which receptors participate in such a way that we can get detectable and useful signals through an amplification process, leading to a useful biosensing or

bioanalytical measurement. We have become interested in this through the use of acetylcholine receptors which have a number of binding sites. The one that we have been working with is the channel site. There are certain drugs and other biomolecules which can selectively or tightly bind to the channel region of that receptor, producing a blocking action. The problem is to take such a binding event and amplify it to a point where this event can be related in an analytical dose-response relationship.

The work that I will describe today deals entirely with the use of PCP as a channel probe. You can think of this system as an analogy to the enzyme amplified immuno techniques which are widely used in clinical chemistry and biotechnology. Think of the receptor as having a binding site for the drug, and when the drug has been conjugated to an enzyme, its activity is altered significantly. We can do this through a substrate reaction, as in NAD going to NADH, which can be followed at 340 nanometers, yielding a kinetic plot where the rate of change of absorbance is related to the receptor concentration. That provides a means of pairing out an analytical procedure, because the substance you want to measure can compete for the sites on the receptor with the enzyme-conjugated drug. As you have an increasing concentration of free drug, you should get a restoration of the activity where the rate increases to a level approaching the original rate. To do this in practice is difficult because there are so many factors to be considered. We get about 50 percent restoration of enzyme activity as the drug concentration is increased in the nanomolar range. The sensitivity range of this technique is already quite superior to many of the other techniques, but not yet as good as radio-receptor assays. To make this into a biosensor, this chemistry can be coupled to an electrode with one or more of the reagents and trapped at the surface of the sensor to make a discrete reusable device. We think, with some development, that it will be possible to make a receptor-based biosensor which would contain this enzyme amplification feature. This would give us a great enhancement in sensitivity.

I'm not sure whether the best strategy in working with chemoreceptor-based biosensors is to employ isolated receptors in some immobilized or recoverable form, or employ the natural structures found in the variety of natural systems where receptors are already held in an optimal environment. We've had success using structures from such organisms as the blue crab from the Chesapeake Bay. The crab waves its antennae about in the water to detect its food, and it does this in real time and can follow a concentration gradient of amino acids to pinpoint its food, even in the dirty Chesapeake Bay. It does it very quickly, very selectively, and repeatedly during its lifetime. We had the idea of employing the sensing hairs dissected out of the antennae of the crabs, and hooking those into a transducer to make a biosensor. This is a biosensor that uses chemoreception but does not isolate the receptors. It uses them in their natural state, and no one, as far as I know, has yet isolated the chemoreceptors from the crab. What you have is a series of neurons or axons which have the chemical sensing end where the receptor sites are found and then a synapse where the electrical signal can be picked up. You get an action potential arising in response to the correct reception event. This can be intercepted and instrumentally used to gather data. We do this under a microscope, using a micropipette microelectrode to contact the

individual nerve fibers exiting from segments of the antennae. Microdissection must be used in making these contacts, but electrophysiologists have been doing this for some time now. We get two kinds of situations. One is the multiunit case in which more than one kind of chemoreceptor structure is contacted, and the resulting signal in response to a stimulus gives a mixture of amplitudes. Such a signal is marginally useful for analytical purposes. If we fine tune the system, making appropriate contacts of our pickup electrode with either a single receptor structure or several that are identical in their behavior, we get the single unit response case where the amplitude of the spikes, the firing of the axon, is uniform and the concentration dependence is reflected in the frequency of those spikes. This is suitable for electronic manipulation and makes an excellent sensing device.

It is possible to integrate the signal and get a dose-response relationship, in this case between glutamate and the integrated signal, in the micromolar range. A better case is one that involves a single unit response which is the constant amplitude where you have exquisite selectivity for the stimulant of interest. This suggests that we can not only make a quantitative measurement, but also qualitatively identify and select out the stimulant of interest. You may wonder how this can be analytically useful; can you get the necessary precision? The dose-response to isoleucine has been treated statistically and has acceptable precision for analytical purposes over a moderate range of concentrations. Using receptors in the natural organism provides additional bonuses over the use of isolated receptors. Two things in particular come out of this kind of system. The dendritic portion of the chemoreceptive cell contains many branches. It is known from electrophysiological studies that the generation of an action potential involves quite a bit of sophisticated noise rejection and anticoincidence circuits already built into the organism. It's how the crab protects itself against false signals. We're able to utilize these advantages in two ways to make an attractive biosensor. For example, a sequence of events that takes place between the stimulus introduction and the final action potential is quite complicated. Two binding events are necessary at a certain time interval in order to exceed a threshold. Only those signals that exceed the threshold are converted into the measured action potential. This permits nature to achieve what is basically an anti-coincidence circuit. One can show in mathematical treatment that the extent of branching of the chemoreceptor axons determines sensitivity. If you have no branches, $N=1$, you have a certain sensitivity range. As the number of branches increases, there is a gain in sensitivity by several orders of magnitude. The typical chemoreceptor structures with which we work have as many as twenty or thirty branches. It's possible with a single antenna portion from an organism like the crab to achieve a wide range of response. Data taken from the blue crab show approximately eight orders of magnitude of response to the stimulant. We have not experimentally achieved the limits of this because we would expect it to plateau off the response of the high concentration in the leveling off to the background of the low. We cannot find that because we cannot make solutions that are reliable concentrations below 10^{-10} molar. But it shows that the use of these natural structures, because of their branching and design, will make it possible to sense

stimulants over a wide concentration range. The time of operation is on the order of milliseconds, so this is a biosensor that can operate in real time. Our measurement techniques are time limiting. We've studied the blue crab extensively because it's readily available to us from the Chesapeake Bay, but more recently we've gone into other systems such as catfish whiskers. It sounds a bit exotic, but these also have the possibility of being used for this purpose.

In addition to the chemoreceptive strategy which I recognize as the main point of this symposium, there are other strategies that are employable for the design of biosensors. We can think of other things such as chemicals or enzymes and other biocatalytic systems as also being molecular recognition elements for biosensor design. The use of ionophores as mediators to measure or monitor chemical reactions was invented in our laboratory. An ionophore, which is a carrier for potassium, can be conjugated to a drug, digoxin, and then incorporated in a plasmic membrane constructed into a conventional potentiometric electrode. This can be employed to detect and monitor the concentration of the antibody to digoxin. Most people are not as much interested in measuring antibodies as they are in measuring the corresponding antigen, and it might be possible to do using this competitive binding approach. Antigen in the membrane reacts with antibody in the sample to give a certain millivolt change. If we then add free antigen to the solution, some of that can react with the antibody in the solution and there will be a competition between the membrane-bound antigen and the free antigen for the antibody. We would get a diminished response, a new delta millivolt which is smaller than that for no free antigen. Recently we succeeded in incorporating this idea into a sensor which has a layered design, using an antibody sensing membrane in contact with a thin layer of entrapped antibody held in place with a collagen membrane. The idea is that the competitive reaction will take place in that space at the tip of the sensor. It has the advantage of not using up the antibody, as the antibody is entrapped and cannot leave, whereas the antibody's antigen can penetrate the collagen membrane. In the test case that we have studied using dinitrophenol, we have achieved excellent analytical sensitivity and precision in the micromolar range. Perhaps more importantly, because the entire competitive reaction is being carried out within the tip of the electrode and not in the whole homogenous solution, this sensor permits us to continuously monitor changing levels of antigen. We have used this probe for antigen monitoring for at least 70 binding and association cycles. Response time is fifteen minutes, typical useful life time is seventeen days, and it's very economical because only a few microliters of antibody are entrapped. Most of this work has been done with special clones of monoclonal antibodies which would be too expensive to employ in a homogeneous system where you throw away the solution after each measurement. Because only a few microliters are necessary, it's possible to work with even the most expensive kinds of monoclonal antibodies.

The third strategy is an old one. The use of biocatalyst is still the most effective means of making a biosensor. The enzyme is entrapped at the surface of a device which could be a fiber optic, or some sort of a thermistor, in our case it's a potentiometric electrode. We have a very selective enzyme catalyzed reaction that converts the material to be measured to a product which is detected by the

electrochemical element. When things are working right this is an unbeatable biosensor. As you introduce additional biochemical components, you are forced to make compromises in the operating condition of those components until the response has been degraded to subacceptable levels. This is the trouble with working with enzyme electrodes. It's possible to employ other natural materials, sometimes very advantageously, for this purpose. In some cases you are able to choose the bacterial strain so that you achieve the necessary analytical properties, such as sensitivity, selectivity, and lifetime. One such case is a culture collection 147. You buy these freeze-dried and culture them in the lab. This makes an excellent biosensor for the amino acid glutamine when coupled to ammonia electrode. There are many other substances present in serum for example, which do not give any response with such a sensor. Instead of using bacterial cells, you might be able to use structures in which the biocatalytically active cells are naturally immobilized by connective tissue. By using porcine kidney cortex cells, it's possible to make an excellent sensor for glutamine with properties very similar to the bacterial sensor but with a much longer lifetime. This sensor was so good that it was used clinically for measurements in cerebrospinal fluids. More recently, it has been found that structures from plant sources can be employed for this purpose. The mesocarp layer of the yellow squash can be dissected out and put on an electrode tip to give an excellent glutamate sensor with a lifetime of at least a week. We've since made plant sensors that are much longer lived. It's interesting to speculate about the use of special structures that are found in nature, like leaves. You can dissect off the outer waxy layer of a leaf exposing the mesophyll where the biocatalytic phase is concentrated. That leaf is then used as a membrane at the tip of a sensor. You don't even need a support membrane because the leaf already has the necessary integrity. In the agricultural field there is considerable interest in measuring herbicides and pesticides in terms of their action upon plants. You can use the tissue of those plants coupled to a sensing element to allow you to measure the effect of the herbicide or pesticide. If you have a plant which is tolerant to a certain herbicide, you know that that plant has a means of breaking down and rendering that herbicide harmless. More recently, we had the idea of using flowers because everybody knows that flowers convert biomolecules into fragrances which are volatile products. That seems like an ideal situation for coupling to a gas sensing membrane electrode. We used a chrysanthemum sepal which is the base where the petals are attached. We took a slice, cross sectioned it, and put it into a gas sensor. You can make a very fine sensor for amino acids such as arginine. More recently we've been working with the Magnolia grandiflora, grown vigorously around here. The argument that I would like to make here is that once you start thinking about nature as a partner in biosensor development, you can find new research ideas everywhere. I feel that the challenge of development of biosensors in the future is not so much the technical perfection of the individual components, but rather the challenge to the imagination. The real challenge is to see what everyone has seen; to think what no one else has thought. Thank you.

Q: Dr. Rechnitz, you mentioned that in your analyte sensor you used special clones of monoclonal antibody. Why were these selected? Was

it avidity or some other property that make them beneficial to that type of sensor?

Rechnitz: You're referring to the antigen amino sensor. We are in the process of exploring the selectivity patterns of different monoclonal antibodies, different clones, with an eye toward finding the best combination of antibody and sensing element. Now, it has not escaped us that this is also a rapid and effective technique for studying antibodies, or the production of antibodies and their properties. With the limited cases that we have investigated so far, we do find differences in the selectivity patterns of different clones of monoclonal antibodies with respect to the response of the sensor.

Eldefrawi: Dr. Rechnitz, in several of your strategies I've noticed that you've used glutamate as a substrate for your sensors. Why glutamate?

Rechnitz: We are generally interested in sensors for amino acids. I used the glutamate examples for my talk so that the substrate would be the same throughout and you could compare the different kind of systems. We have studied all of the 20 essential amino acids and numerous other substrates, but I just chose that for the purpose of this presentation so that we would have something that's being held constant.

Eldefrawi: I'm very much intrigued by the glutamate receptors in the brain. Were you considering specific recognition of glutamate that could be eventually applied to a receptor?

Rechnitz: I think we could certainly do this. We have more recently gotten heavily into nucleotide responses and we're interested in the hormones as well.

Diamond: Professor Rechnitz, you pointed out that enzyme electrodes have been around for many years and there are serious problems in terms of commercialization. Do you think that there will be similar problems with some of the approaches that you've talked about with the amino sensors and the receptor-based sensors of those natural materials? For instance, with if you make five different sensors, how do they respond?

Rechnitz: The enzyme electrodes are a very instructive example of the kind of the practical obstacles that exist. My first paper with enzyme electrodes was published in 1962, and we still don't have many examples of commercial cases. There are a few now, mostly for glucose. It's taken a long time to carry that idea forward into a practical device that will stand the demands of real life. We make absolutely no claim regarding the practical exploitation of the concepts that I have tried to present here today.

Valdes: You stated in your talk that, as you increase the number of dendritic branches in crab antennae, you increase sensitivity. To use nature in a practical manner, how would you control the total sensitivity and calibrate the sensitivity of one sensor made out of one antenna to another sensor made out of another?

Rechnitz: I think that's a real problem. Calibration isn't really so difficult to do, but there are practical obstacles even worse than the one to which you're alluding, and that is lifetime. When we first started, we could only run one of these for about fifteen minutes, and now we're about up to forty-eight hours. That's not enough for a practical device. We have seen literature where people in the marine sciences have managed to keep natural receptor structures viable for up to one hundred fifty days. We're trying to apply some of those profusion techniques to our kind of work.

Patton: Dr. Rechnitz, do you think that the natural membrane electrodes may serve as models of what's going on within them at a molecular level, to find out if they're multi-enzyme type of mechanisms? If so, can we isolate the multiple enzyme cofactors so that we can achieve reproducibility and manufacturability?

Rechnitz: I have felt for some time that there are unexpected benefits that come out of this research which have more relevance to people who are interested in the biochemistry of the systems than for someone who wants to make a practical biosensor. We were able to confirm and identify the existence of an enzyme in a magnolia blossom which had previously been known only in bacteria; this was of interest to plant physiologists. In working with the leaves and the herbicides, it was possible to use this technique as a means of elucidating some of the biochemical pathways. These are some of the unexpected side benefits that come out of this research. There's no way to know which will be more important in the long run. When designing sensor systems, you have to worry about noise, sensitivity, responsible dynamic range of signals and adaptation. It may be a great idea if we, by looking at these systems learn something. A classical example of such a system is the optical sensor. A lot of biologists have spent the most time to understand the retina, and it's one of the most understood systems in human beings. Right now, electrical engineers have begun to produce the electronic distance of your systems and can function within one percent to two percent to perform some functions of the retinas. The advantage of this system is that you do have a lot of signal processing on your sensor area, and a lot of intelligence built in the sensor itself. There is a trend to go in that direction in building the general system processing system.

Patton: Next we will move into optical sensing technology and hear two interesting papers on optical waveguides. Our first speaker is Myron Block who is president of OZD, Incorporated.

Block: We didn't follow the admonition of the previous speaker to work on something that everyone else has seen. We chose instead to work on a rather obscure phenomena- an evanescent wave photo sensor. First I want to acknowledge the contribution of the late Dr. Tomas Hirschfeld who is co-inventor of this technology. After talking about the physical principles on which the sensor operates, I'm going to turn over the talk to Tom Glass who will describe the hardware and some initial data.

The sensor was originally developed for immunochemistry and immunoassay, and the data we have is from Ciba Corning. Their objective is to put this in a doctor's office. I will try to explain the optical principles from a heuristic point of view; that's for the benefit of the biologists and probably the amusement of the physicists. Probably the easiest way to describe this phenomena is to describe what is called total internal reflection. It is called total because it's a lossless reflection. The expense of putting prisms into binoculars was chosen instead of a mirror because a prism is more efficient than a mirror, there is no absorption in the metal coating, the reflectivity is a higher percentage, and it does well with age. Light comes down, is reflected from a face and comes out. There is a penetration into the less dense medium even though it is supposed to be total internal reflection; not everything is internal, something must be external. If the light wave did not penetrate it would have no way of "knowing" that there was air on the other side. That penetration is called the evanescent wave. The thickness of the evanescent wave must be less than a wavelength, because if the evanescent wave were a wavelength or greater, then it would have a grip on the boundary and be able to propagate into the less dense medium. So the conditions for propagation are not there. We have two properties of the evanescent wave: it is thinner than a wavelength and it exists outside in the forbidden medium. There are a lot of other properties of the evanescent wave that this heuristic argument doesn't address but we will leave those out for the moment.

It turns out that the intensity in the evanescent wave is greater than the intensity in normal propagation. The fiber has a certain finite width, and acts as a pipe which carries photons along at a certain rate. They get into the evanescent zone and now the pipe dimensions are compacted down to less than a wavelength, raising the density of photons. When you do the calculations and go through the wave equations, the electric vector intensity in this region is a factor of ten greater than it is in normal propagation. What we do is put fluorescent material within this sub-wavelength sized zone. The fluorescent molecules are excited more efficiently than they would be under normal circumstances in normal fluorometry. What happens to the fluorescent light in this region? Well, you would think that being in the air as in a prism, the fluorescence would take place in all directions. To some extent it does. During the actual process of emitting that wave, its radiation pattern is modified in such a way as to produce most of the fluorescence in the evanescent wave direction, which again gives us an advantage in studying fluorescence.

We've chosen to use this obscure phenomena, for the following reasons. Because we are working with an evanescent zone which is less than a wavelength, we have an optical method of separating bound from free. Anything that's outside of the region isn't seen, so that only things that are bound on the surface are seen. The other reason we've elected to do this is that it's an excellent way of doing a fluorescence assay that is very sensitive. We believe that it has the potential sensitivity of being able to see down to a few hundred fluorescent molecules. By using a fiber instead of a flat plate, we are able to encompass the fiber in a capillary tube and then we become sample-volume independent. As long as the manufacturer coats the fiber with a reagent to a proper fixed length, any reagent overflow doesn't

affect anything. We are able to do very quantitative immunoassay and not have to go through the problems of measuring out sample volumes or preparing samples. This works in whole blood. The other thing is that the geometry of using the fiber increases speed. The diffusion to a line is much faster, almost at homogeneous speeds, than having to diffuse to a flat plane. Those are the general advantages. I will turn this over to Dr. Tom Glass. Thank you.

Glass: I first want to explain how the sensor operates. What we have is a fiber optic. The excitation light is put into the fiber and guided down its length. The sample is outside. Molecules that are within their evanescent penetration depth absorb part of the excitation and become excited, while molecules that are outside are not excited. Fluorescers inside the evanescent zone can emit evanescent photons. If a fluorescer is in the bulk of the solution, it emits light if it is excited, although in our system it is not, and that light cannot become trapped radiation in the fiber. These rays are transmitted out again--not as bound modes in the fiber. A fraction of the fluorescence that the surface of the fiber emits will tunnel into trapped modes on the fiber, propagate down the length of the fiber and exist again for detection. We can use this in a competitive assay. The sensor geometry that we have demonstrated consists of a fiber, with an entrance cone of excitation light. The tunneled fluorescence comes back out in a cone we detect at the same end. We put the excitation into one end of the fiber, and we detect the fluorescence at the same end. It gives us an advantage over working at the other end in the background level since we don't have as much excitation light against which to discriminate. One of the first questions that comes to mind is how far does this evanescent wave extend? How far apart can the molecules be and still be excited? In particular, can a fluor on a fairly large molecule, such as a receptor, be excited in this manner? The electric field in the evanescent zone is an exponentially decaying field and the DP that Myron showed you earlier is the $1/E$ point for the electric field amplitude. It's further complicated by the fact that this picture applies only for a single angle of incidence; the ray comes in at a single angle, you get a single exponentially decaying wave across the interface. What we have in our sensor is a cone, so we have a wide variety of angles of incidence. Then we add up all of the exponential decays to get a true effective penetration depth. The depth of penetration as a function of angle of incidence is DP divided by λ , so these are in units of wavelengths. If you fill a cone up to and including the critical angle, you will get a depth of penetration for excitation which will be over 0.2 wavelengths and certainly less than 0.5 wavelengths of penetration. So we work in the visible and in the middle visible; that gives us one hundred fifty to two hundred nanometers of distance over which we can excite molecules. Because we use our sensor in an evanescent collection mode as well, we have another factor that has to be considered, and that is the efficiency of collection of the emitted fluorescence. Under an SBIR contract from CRDEC directed by Dr. Valdes, we calculated the signal intensity as a function of distance from the waveguide surface. At five hundred nanometers, we have the signal intensity dropping off in distance; you are down by one wavelength. However, this isn't so bad, for the " $1/E$ point" is not a true exponential. Roughly 36% is around

eighty microns of penetration depth which looks adequate for most receptors. The other factor to consider in terms of sensitivity is the size of the cone of excitation. The evanescent penetration increases dramatically with angle as you approach the critical angle. Also, the electric field intensity is stronger at the critical angle, both of which contribute to the intimate sensitivity in terms of detection. In work that we did under contract to NASA, we explored that question in terms of numerical aperture which represents the size of the cone. In the cone of excitation light, numerical aperture is the sine of the half angle. We were able to show that the signal level increases approximately between the eighth and the ninth power of the numerical aperture. So, for sensitivity, it is very important in instrument and sensor design maximize the numerical aperture.

We have been through several generations of instruments by now and our latest one consists of a six watt incandescent lamp, some optics to image the filament onto the fiber face, a filter for fluorescein dye, and a spatial filter so that the filament is spectrally filtered and then imaged onto the fiber face. The chemistry of binding takes place on the outer surface of the fiber, fluorescence comes back out the end, is collected by the lens, passes through the dichroic beam splitter, and is further spectrally filtered to reduce background, and then imaged on a silicon photo detector for detection. This instrument fits in a suitcase. The lamp is in one area, filters, dichroic, and the fiber lay in another area, and the detector is in another plane along with the amplifier associated with the detector. We can detect about 10^6 molecules of fluorescein. We've also miniaturized the instrument. Our first generation miniature instrument is a working prototype. Under the same contract with Dr. Valdes, we explored optimum dye-LED combinations. We had to use a LED source to get the size down. With that dye, in one of the suitcase size instruments modified to hold the red LED, we are able to detect about 10^4 molecules of dye. I'm comfortable with being able to go to a thousand and I think we might be able to push it to a hundred molecules. That involves things like cooling the detector against electronic noise, temperature stabilizing the LED and pulsing it to get more power out of it.

I would like to acknowledge Ciba Corning Diagnostics for the following immunoassay data. The data for a ferritin assay is shown here in nanograms per ml which well spans the clinical range for that particular drug. The time axis is in seconds so what we are seeing at 600 seconds is the binding of the labeled antibody. This is taken in a one-step sandwich format where the fluorescently labeled antibody is mixed with the analyte solution and then added immediately to the fiber and allowed to react. As the antibody accumulates on the fiber, the signal increases in time. In as little as a minute, we have good separation of the various concentrations of analyte. What they've done is constructed a standard curve based on the rate of accumulation of signal over the first forty seconds after adding it. This is compared to Corning's commercial MO phase radioimmunoassay. There is some difference in response but both are certainly capable of differentiating the analyte.

Q: The launching of the evanescent wave involves much more than simply illuminating the end of it, so isn't there a fundamental difference

between a conventional fiber optic illuminator and something which can launch and transmit an evanescent wave?

Glass: There is not a fundamental difference; however, there is an important practical difference and that is in the numerical aperture. It's very important for maximum sensitivity to be sure that you have the maximum numerical aperture, but often in fiber optic illuminators they are not optimized for that application. In fact, in communications the numerical aperture is typically limited by the fiber to a much lower value. The index of the material surrounding the fiber, along with the index of the core of the fiber, limits the numerical aperture that you can insert.

Q: That leads to the second question. Does the nature of this preclude long distance transmission, as through several meters, of a clad or other fiber to a section which will then permit an evanescent wave to appear?

Block: If you had a way of keeping the fiber protected so that it operates in air or water the whole length, then you could go as far as you wanted; otherwise you throw away sensitivity.

Q: Does that mean that one would be clad down to the section covered with the selective reagent?

Block: If you clad down to that region, you can't deliver the numerical aperture because it is limited by the clad region. Water has a lower index than the plastic, so it's the region of lowest index that sets your maximum numerical aperture.

Q: So it is not going to be possible to remote the electronics and everything from the actual sensing zone. Do those two have to be relatively close?

Block: Not necessarily. There are some efforts and some are thought to have very low index cladding material.

Q: You send a wave, you excite the luminescence and then you send back the light. How much light do you get back? Why can't you do it on a glass plate?

Glass: It can be done with a glass plate. There are advantages to the fiber because in the circular symmetry the light is confined to two dimensions, and in a plate geometry it's confined to one. It can spread out along the plate, but that makes efficient collection difficult.

Q: But you use some optics to collect the light from the fiber optic and focus it in a large area photodiode.

Block: In optical instrumentation, the important thing is specific intensity. If you are illuminating this room, it's flux. But to activate film or to activate detectors, you need intensity; and if you have the big spreadout area of light, it means that you can't focus

down on the small detector area. The trapped mode or the trapped feature preserves brightness. It's one of the rare cases where the longer you make the sample cell the higher the signal. In most sample cells you increase the length of the sample cell and you start to get self-absorption. Here you don't get self-absorption. The intensity is what matters and our system preserves intensity.

Q: You don't use a laser. If it's a laser, then it is much easier to recover the light and the fiber rather than with LED.

Glass: Lasers have wavelengths that are too long for the dyes about which we know.

Block: If some chemist would give us some dyes we would love to use a laser.

Q: What kind of LED do you use for this work?

Glass: It's at 660 nanometers.

Q: You mentioned a sensitivity example of 10^6 fluorescein molecules. Presumably that's in a small evanescent volume. In practice, what kind of concentration is that?

Block: The instrument sensitivity is about 10^{-14} noise equivalent concentration in molar.

Glass: That's not for fluorescein, that's for phycobilic protein. It's between 10^{-12} and 10^{-11} molar fluorescein solution. That's a noise equivalent concentration meaning that gives rise to a signal equal to the noise.

Block: If you permit binding, you enhance that by a factor of a thousand. That's the solution, although it depends on whether you are talking about the concentration of the capillary or the concentration on the fiber. The fiber essentially concentrates, and ideally all the material will diffuse from the capillary into the fiber. And so you pick up a factor of a thousand because the ratio of the volume in the capillary to the volume in the evanescent zone is a factor of a thousand, which helps reduce the background effect.

Q: Do you have any problems with nonspecific binding or absorption of fluorescent problems with nonspecific binding or absorption of fluorescent labelling?

Glass: Yes, some can happen. We run a control:

Q: It seems that that would be accentuated as well because you are looking at a very small surface area.

Glass: Any fluor that gets into the evanescent zone we see very well.
Block: Corning's effort has been primarily in the area of coating the fibers, the effect on nonspecific binding, and the effect on CV's, because their plan is for a quantitative assay. The program is out of

the research and development stage and is now in engineering for product configuration for the doctor's office. I've tried to get them to share their fiber coating techniques which they've developed but I don't think they've ever shared that information. On special cases they've said if you send us the reagents, we'll coat them for you but we won't tell you what we do. Their reporting CV's for commercial and diagnostic range from less than the five percent range.

Glass: Certainly less than ten percent.

Patton: Nonspecific absorption on any type of glass surface is a problem, particularly a wave guide in which you are interested in what's happening optically on the surface. I do believe that the nonspecific absorption problem can be solved.

Q: Does anything in this technology preclude the employment of the flow cell incorporating the capillary you described in the flow cell?

Glass: No. That's how we do it. We do it also strictly with capillary filling. All of the work that I showed was done with the flow cell.

Patton: I'd like to introduce Professor Joe Andrade who's dean of the college of engineering at the University of Utah.

Andrade: I would like to continue the same line of discussion and address the question of how one might take the sensor that was just described and try to make a continuous and possibly even a remote device out of it. You might want to subtitle the talk "Some Unsolved Problems and Concerns in the Development of Receptor-Based Optical Biosensors." Our group's goal is remote semicontinuous optical biosensors based on fluoroimmunoassays, not disposable one-shot devices. There is considerable interest in the biotechnology and biochemical engineering business for an on-line process control of biochemical processes. There's some interest in waste and water treatment water monitoring, and considerable interest in medicine and diagnostics. It's possible that this can be done in an in vivo implanted sensor mode and biomedical research as well. There is some interest in the defense establishment in continuous on-line monitoring. Our group's basic science goals are protein biochemistry and how that couples with surface and interface chemistry, with optics and spectroscopy to probe that. Polymer chemistry in surfactants are used to modify it. Our engineering goals are to employ proteins as engineering machines and devices.

The area that's been the most developed in the general engineering community is sensors, biosensors in particular. How does one detect and monitor one of the proteins at an interface, in a reproducible quantitative manner? Almost any labelling process can change the physical chemical characteristics. About five years ago we were starting to look at probes of proteins at interfaces, and decided to would look at the intrinsic fluorescence which is present in most proteins, primarily via the tryptophan amino acid moiety. That absorbs in the UV and fluoresces a little closer into the visible, with a respectable quantum yield of about ten percent. Most proteins of interest are intrinsically labelled with a UV fluor. There's

considerable information available from fluorescence in addition to the intensity and spectral information. From fluorescent and depolarization information, fluorescent lifetimes can be deduced and one can measure energy transfer and use specific probes to change some of these other parameters. There's a wide number of information channels to fluorescence. We use a dove tail prism in the research apparatus and a fiber optic device in the analytical and engineering apparatus. Our device is equipped with a flow cell because we want to control the hydrodynamics and be able to follow the mass transport diffusion limitations and related considerations. On the other side of the flow channel, a gamma detector is mounted so we can use labelled proteins and use the radiolabelled signal as a calibration. The total internal reflections fluorescent signal is similar to the buffer background. As one injects protein solution into the cell, one sees a component of the bulk solution. The evanescent wave and the visible wave penetrate on the order of 2000 angstroms, and in the UV about 1000 angstroms. The protein's dimensions are of the order of about 100 angstroms. You see what might be called a boundary layer or evanescent volume component.

Protein absorption at an interface is kinetically a relatively slow process and takes on the order of minutes to hours to reach saturation. This is a non-specific binding process. At the end of that process you remove the protein solution, the bulk signal disappears, and you can see if the absorbed layer is stable. Is it reversible or irreversible? Normally, you find some component of various populations, some of which will be removed or desorbed into the buffer. The interface optics are very well known, particularly if you have a system where the evanescent modes are well defined. In the total internal reflection prism geometry, there's a single well defined mode. The electric field is decaying exponentially into the low refractor index phase. The fluorescence being emitted is the integral under that and involves a quantum yield, the absorption coefficient of the fluor, concentration of the fluor, the proportion of the fluorescence emission which is collected, the square of the electric field strength, and so forth. One can rigorously quantitate that, if you assume a concentration profile of the interface. We assume a step concentration profile which Myron Block indicated was due to normal absorption or specific binding processes. The protein is concentrated at the interface by a factor of as much as a thousand or more over its concentration in the bulk, and then we assume a uniform bulk concentration profile. You can ratio those two profiles, make a major assumption that the quantum yields of the protein in the layer at the interface and in the bulk are the same. That's a bad assumption in many cases, particularly for the intrinsic fluor. There's another problem in the ultraviolet, which is scattering due to the imperfection of the optics. The optics are never 100 percent perfect, not even an optical fiber, or the fiber solution interface, and so there is a component which does get scattered into the far field. That component propagates into the cell and generates fluorescence from the bulk solution, so the optical trick of evanescence allows you to separate the bound from the bulk through the fact that the evanescent wave is constrained or contained at the interface. There can be some excitation of the bulk through this scattering phenomenon. In the ultraviolet, that is a particular problem because scattering occurs as

the inverse of the wave length, so when we use ultraviolet detection for fundamental studies, we have a scattering problem which is an order of magnitude greater than when we're in the visible. If you plot the fluorescence as a function of concentration of something with a very high absorption coefficient, you find that the scatter signal rapidly plateaus due to inner filter effect. You never see an inner filter effect because it's essentially linear all the way to saturation or precipitation of the solution. By playing that standardization game, one can sort out the scatter component from the evanescent component, which allows very rigorous quantitation even in the ultraviolet. The advantage of working in the ultraviolet has to do with the interfacial biochemistry to understand what is happening with antibody labelling and antibody immobilization with the kinetics of antigen binding.

It was clear to us about four or five years ago that, if we could detect one protein interacting with and interfacing with another, we could also detect two. That led to our interest in applying total internal reflection fluorescence which was patented by Hirschfeld in the 60's as an analytical tool in immuno assays. One can do fluoroimmuno assay in the ultraviolet; it's not particularly recommended, but certainly antibodies are UV fluorescent and most of the protein antigens of interest are also UV fluorescent. The count rates are low, UV light sources and UV detectors are still a problem, but the biggest problem is that you fry the immobilized component after significant excitation time in a sort of oxygen containing solution. That's not amenable to our long range goal of a continuous or semicontinuous remote sensor. The modes of operation of such a sensor have already been described. There are a whole variety of ways to do it, whether a saturation type assay or competitive binding assay, one can monitor antigen, antibody, and so forth. I'm going to focus on immobilized antibody for antigen or hapten detection. One of the big concerns is how to immobilize the antibody. In conventional, one-shot diagnostic immunoassay, that's often done by physical absorption onto a polystyrene or other support. That can work very well. We've looked at IGG physical absorption on dimethyl dichloro silanized quartz, on unmodified or hydrophilic quartz and on amino propyl silanized quartz. Our experience is that amino silane is a very bad support on which to immobilize and leads to fairly significant changes. The DDS is not too bad, as it is a sort of a model hydrophobic surface which one can covalently immobilize. We do it through a preabsorption of albumin followed by a glutaraldehyde cross-linking of the albumin and a glutaraldehyde coupling and cross-linking of the antibody. We've also tried glutaraldehyde coupled directly to an APS and cross-linked the antibody that way, and we've also played with protein A in order to try to orient the antibody. The model systems that we've used are an anti-digoxin system, a monoclonal to digoxin, and a commercial goat IGG to human IGG antigen. The binding constants in solution have not changed dramatically on binding or immobilization to a glutaraldehyde support or on physical absorption to a DDS support. In some cases there can be a significant change in the binding constant, but not in these model systems with which we've chosen to work.

We are interested in the fiber optic sensor approach and we use a large fiber with a silica core, where the cladding has been stripped in the sensing region. There are really three fundamental problems in making such a technology suitable for remote continuous use. It's

clear that fluorescent reagent is required. There are ways to get around that, but they all have some problems. How do you deliver a fluorescent reagent for a continuous remote sensor? One could have a plumbing system or perhaps a liposomal kind of system, to deliver reagent remotely. We've chosen to approach it from a photochemical point of view by using nitroaromatic linkages which are intrinsically photolabile. They break when they absorb photons of 350 to 360 nanometers, so we're coupling those linkages into a polymer. This polymer is actually a hydropropylmethacrylamide derivative which has been used extensively as a drug carrier for cancer treatment in experimental animals. One is a side chain to the basic polymer structure, another side chain contains a nitroaromatic group with a reactive amine, which can then combine the fluorescein labelled antigen or antibody. That seems to work reasonably well in bulk solution. The project now is focusing on trying to make a gel which can be interfaced with the optical fiber in such a way that a pulse of 316 nanometer light down the fiber will then release a pulse of fluorescently labelled reagent. The more serious problem is: are we running a sensor or a dosimeter? Most of what's been discussed during the meeting really involves dosimeters. If we pull out the stops on sensitivity and use systems with high binding constants, then for all practical purposes that binding is irreversible. It clearly can be displaced and so made "reversible", but it takes rigorous conditions or much patience in terms of response time to do that. It's both an advantage and a disadvantage of antigen-antibody systems. The on-rate constant, forming the antigen-antibody complex, and the off-rate constant are such that you get a high binding constant. You want a very high binding constant because that gives you a high sensitivity, but it also means that the off-rate is very slow which means that the response time is very slow. We would like to cut that down and have a short response time, but that is at the expense of the binding constant which means the sensitivity drops.

The ideal system is one with a high binding constant during the measurement and a very low binding constant between the measurements. We'd like to be able to use it as a dosimeter during the measurement, and then rezero it between measurements. Some of you have genetically engineered systems which may be able to do that. Can we change the local environment around the antibody to change its binding thermodynamics? We've chosen to look at three approaches to that, but one is going to be very hard, one will be relatively easy, and the other, the most interesting one, will take a few years. One approach is to run the appropriate solution through an antigen-antibody column, and if the solutions are appropriate, you can displace the bond. You do that by changing the conformation of the antigen, the antibody or both, or changing the nature of the media so the antigen antibody interaction is significantly modified. That's a solute approach; you can do it through change in pH, through change in the hydrophobic nature of the hydrophobic structure breaking agents or others. We would like to have a reversible solute in order to avoid plumbing. You can do it by plumbing; simply plumb the system and wash it out with the appropriate elute, but we'd like to avoid that in a truly remote sensor. Our approach is to make a macromolecular switch which, under light, will induce a photoconformational change. We try to get a coil expansion, which basically takes a molecule and pops it out so it's co-

immobilized with the antibody, but under light it expands and now is essentially ruining the surrounding solution. It's a concentrated solute, but when it's finished we can pull it out of the way by simply pulling off the light or exposing to light of a different wave length to change the conformation. Our idea is to have a solute which we can direct into or out of the binding region. The model system we've chosen to work with for this is a series of monoclonal antibodies to fluorine. It's probably the most widely used dye in fluorimmunoassays. The other good aspect of using this as the hapten is that the fluorescence of fluorescein quenches when it binds to its specific antibody.

It's very easy to do an on- and off-rate assay and to measure the antigen-antibody thermodynamics with this system. The binding constant of different monoclonals changes at different rates with temperature, so we can choose the right binding constant for that particular experiment. Since you have the temperature-dependent data, you can extract the thermodynamics from that. The entropy-enthalpy compensation process leads to the free energy being relatively constant until you get to temperatures at which the molecule begins to irreversibly denature. You can expand that free energy curve and show that there are some significant changes in free energy with temperature. One good aspect of this system is that you can not only measure the overall constant but also the off-rate constant or the dissociation lifetime. If we were operating at about 10° C, it would take about 5000 seconds for this thing to dissociate or reach a level of equilibrium with a change in antigen concentration or fluorescein concentration. At body temperature, that's down to the order of sixty or seventy seconds. It's clear that we can change a dissociation lifetime by changing temperature, at least in many antibody classes. How do you change temperature at the interface without frying the solution? We haven't done this yet with an evanescent wave. Coming in with a near IR beam, water has a fairly strong absorption at about 1.4 to 1.5 microns, and a much stronger absorption at 2. There are good, portable light sources available in that region. The solute approach is an interesting one if we look at the log of the binding constant as a function of the concentration of a particular solute. In this case it's methylpentanediol which is commonly used as a crystallization solvent by x-ray crystallographers. With methylpentanediol the change in the solute concentration drops the binding constant in a substantial manner by up to three orders of magnitude. If we could have a solute similar to that incorporated into a polymer which could be photo changeable, then we might have something. The hydroxypropylmethylacrylamide monomer worked slightly better, it went up to about eight percent, where the other went up to about 50 percent solute. In this particular case, by adding about eight percent weight by volume of the monomer, we dropped the binding constant by an order of magnitude. It does appear feasible from an antigen- antibody thermodynamic point of view to do what we've suggested here. We can try to design a polymer which can be immobilized or co-immobilized at the interface together with the antibody, and have a region which can expand under optical illumination, and as that expands, it drives a concentrated solute into the binding region. The solute is reusable.

The photoconformational system we've chosen, azobenzene is found in literature going back to about the early 1940's. Azobenzene

is essentially nonpolar with no dipole moment in its normal ground or trans state, but when excited with 350 to 360 nanometers it pops into the cis form which has a fairly substantial dipole moment. Polypeptides with azobenzene side chains can undergo significant changes in the alpha helix content as a result of this change in polarity of the side chains under optical stimulation. It's a copolymer approach, and again we used hydropropylmethacrylamide to give us the water solubility and the general compatibility, and an azobenzene derived hydroxymethylacrylamide monomer to give us this photo conformational property. A small amount of monomer with a reactive side chain allows us to couple to the surface or to antibody or antibody fragments. We started out with methacrylic acid because the change in the polarity of this group due to the trans-cis isomerization changes the pK of this group, which changes the degree of the ionization, which changes the coil size through a polyelectrolyte coil expansion argument. We do see changes in the pK and in the degree of ionization as a result of the photoisomerization, but that's not sufficient to this point in the design of the copolymer to get a significant coil expansion because the methacrylate backbone is extremely hydrophobic. Its hydrophobicity is sufficient to overcome that. We're now moving to an acrylic acid system for the charge component and other components to try to minimize that hydrophobic backbone. We're fairly confident that it will work eventually. It's also obvious that a random copolymer is not likely to work in practice. We're going to have to go to an asymmetric polymer. It's essentially a block system, where we have an immobilization block, the coil expansion photoconformation block and the solute delivery component.

A couple of other projects at which we're looking are of some interest. Light sources which minimize the fluorescence background of serum have a strong use in continuous remote sensors for use in blood and in medical application. It's clear that if you excite much below 500 nanometers you pick up a significant fluorescence from serum. What we've done is, rather than go to the phycobilic proteins in red LED's, we're playing with the new generation of helium neon lasers. A green helium neon which puts out about 543, and relatively inexpensively one could pick up a milliwatt or so at 543, and the rhodamine dyes which have many similarities to the fluorescein in some respects, absorb and emit very nicely as a result of that excitation. That's a direction in which we're going on the sensor side. Another unresolved problem that's been alluded to is this whole question of nonspecific binding. There are many possibilities for nonspecific binding. First of all, there are always parts of the interface that are unmodified with protein, in the case of the classical silane reaction, there are pieces of the interface which may be unsilanized, and one can get physical and nonspecific binding to the support. The antibody or antigen or hapten that you're immobilizing is being immobilized through some chemistry. Dr. Patton referred to a urethane-like linkage in his talk. All of those will become involved in nonspecific binding to various extents, depending on what A, B, C, and D, are in the media. There's nonspecific binding to the antibody itself, because the immobilization results in a slight change in conformation because it's present in a different microenvironment than it was out in the bulk solution. There can be nonspecific binding to the antibody or cross-reactivity with other material.

Our group has been interested in biocompatibility in the blood environment for about twenty years, and we've been very interested in the whole question of protein resistance surfaces; that is, how do you keep proteins off an interface? The only approach that we've come across that seems to be general, nonspecific, and works, is to immobilize polyethylene oxide to the surface and other neutral hydrophilic polymers. Polyethylene oxide appears to be unique. The argument is an excluded volume steric exclusion argument. PEO is used extensively in a colloid field to sterically stabilize colloidal dispersions, and that's one of the reasons that it works. A protein, in order to adsorb at such a surface, has to compress or minimize this excluded volume. You might think of compromising the configuration entropy of the PEO chain. That is an undesirable process unless there is a strong interaction between the protein and that particular surface. That's a good nonspecific repulsive technique. We're now immobilizing our antibodies through PEO tethers, so that at least the PEO tether will be reasonably resistant to nonspecific binding. Then we're trying to quench or cover the rest of the surface in and around the antibody with low molecular weight PEO chains, to minimize any other nonspecific binding.

Some of the surface chemistry and biocompatibility concerns we're addressing, then, are fluorescent reagent delivery, remotely under optical control, modulation of antigen-antibody binding constants both thermally and through photoconformational changing, or photoexpansion of an appropriately designed polymer to be co-immobilized with an antibody. The question of optimizing antibody orientation is immobilization, and our approach to that is through polyethylene oxide to get the antibody away from the interface using a fully hydrophilic tether. In essence, to keep it in a microenvironment as similar to that as bulk solution as possible. We're working on minimizing nonspecific binding through steric exclusion arguments, largely using polyethylene oxide approaches, and the model systems that we're working with at the IGG, anti-IGG polyclonal. A digoxin, anti-digoxin monoclonal system was provided by the University of Utah and more recently fluorescein, anti-fluorescein system from Illinois and Utah. We're moving into sensors for coagulation proteins, again sponsored by NIH, and the systems we've chosen to work with initially are thrombin and anti-thrombin 3 and their appropriate monoclonals.

Block: Your work with the Greeley source looks very interesting. Just for comparison, all we're dumping on our system is a couple microwatts. You get up to a milliwatt. I think you'll win the prize for the lowest detection level before we get there, because you'd have factor of a thousand immediately, so you'd be able to get to hundreds of molecules easily.

Andrade: We haven't attempted to do any optical optimization as you have, and so our numerical apertures and these things are all suboptimal.

Q: You mentioned the polyethylene oxide tethers. How do you attach the polyethylene oxide chains to the solid surface?

Andrade: There are a variety of ways one can do that. Most of what we've been doing involves the aminopropylsilanization of silica and in using that amino group for other coupling reactions, so the PEO can be derivatized. You can even buy diamino PEO. We are producing PEO with two different functional groups, one with different groups on each end so we can be sure to minimize looping effect.

Q: Are you using a silane?

Andrade: As far as the activation of the silica surface, yes.

Eldefrawi: I would like to make a follow up comment on the sources and ways to get the light. Another approach may be active fibers, which are getting very popular. They're beginning to emerge as commercial products. Your technology has an active fiber that you can pan in one wave length and you can get another wave length.

Andrade: You mean these fibers containing a built in fluor?

Eldefrawi: Terbium, yes.

Q: You mentioned that you're going to cover the rationale leading to the photoexpandable fibers. Could you elaborate on that?

Andrade: There's an old effect in the polymer field called the photo viscosity effect. If you take methacrylic acid or acrylic acid systems, basically polyelectrolytes, and heavily derivatize them with azobenzene derivatives, you can show the viscosity changes dramatically in the presence of light. You can also, in the presence or absence of light, titrate and get the pK of the systems. There's no question that in the appropriately designed polymer that happens in solution.

Q: I could explain a change in viscosity as a change in electronic configuration leading to a change in hydrogen bonding, for instance.

Andrade: Yes, but they're getting viscosity changes of various significant amounts.

Q: You spoke about data where a binding constant enthalpy was a function of temperature. Maybe it's something peculiar to the biochemistry, but chemistry enthalpies don't change with temperature typically. Free energies do by the change in the entropy, component but not the enthalpy.

Andrade: Most of it is based on an analysis that has gone through a whole variety of ligand receptor binding systems.

Q: What that implies is that the bond strength changes with temperature. I don't know too many examples of that.

Andrade: A number of classical bond types in biochemistry do change with temperature.

Patton: Thank you very much for your paper. From where this conference was two years ago, we've come a long way. As I look back on the day, it sounds like wonderful things and exciting things are happening in specific areas: Transducer technology, optical waveguide technology, new solid-state chemfets, and inductive devices. In the big advances in the individual components of what one needs to make a biosensor transducer, technology is moving along. In areas where we know what the receptors are and how they work, many exciting things are happening. We are back to the basic question--how much do we know about membrane receptors themselves? Do we know enough to be able to bring them in, make them up with transducers, and expect the same kind of success that we've had with other receptors like antibodies and enzymes? That's where the question is. From what I've seen in the past two or three years, there has been real progress in both areas. The papers I heard yesterday were encouraging and as a chemist even I could understand part of it. More of the mechanisms of how the receptors are operating seem to be understood. I think this technology definitely has a bright future.

Valdes: I agree that, in the past couple of years this field has come a long way. The first biosensor conference was very small and was limited to contractors. When we first designed the program it had very limited backing at CRDEC, but the program is ranked as one of the top Army research priorities.



DEPARTMENT OF THE ARMY
US ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
EDGEWOOD CHEMICAL BIOLOGICAL CENTER
5183 BLACKHAWK ROAD
ABERDEEN PROVING GROUND, MD 21010-5424

REPLY TO
ATTENTION OF:

09 APR 2013

RDCB-DPC-RS

MEMORANDUM THRU Technical Director, Edgewood Chemical Biological Center (ECBC)
(RDCB-D/Mr. Joseph D. Wenzand), 5183 Blackhawk Road, Aberdeen Proving Ground, MD
21010-5424

FOR Office of the Chief Counsel, US Army Research, Development and Engineering Command
(RDECOM)(AMSRD-CCF/Ms. Kelly Knapp), 3071 Aberdeen Boulevard, Aberdeen Proving
Ground, MD 21005-5424

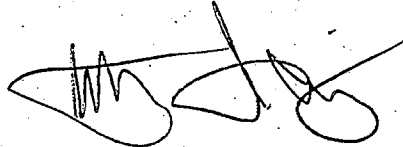
SUBJECT: Operations Security/Freedom of Information Act (FOIA) Review Request

1. The purpose of this memorandum is to recommend the release of information in regard to RDECOM FOIA Request, FA-13-0027.
2. The ECBC received RDECOM FOIA Request FA-13-0027 from Ms. Kelly Knapp, RDECOM FOIA Officer. The request was for an Operations Security review of documents from the Department of the Army Inspector General's FOIA Office which originated from Paige Tomicelli of the International Center for Technology Assessment.
3. A review of the below requested documents was conducted by an ECBC subject matter expert:
 - a. ADB-113338 (CB-000027), Possible Application of Biotechnology to the Development of Biological Agents by Potential Enemies, dated June 1987.
 - b. AB-117238 (CB-00675), Final Report of the AD HOC Sub-group on Army Biological Defense Research Program, dated July 1987.
 - c. ADA-198966 (CB-001819), Third Annual Conference on Receptor Based Bio-Sensors, dated July 1988.
 - d. ADA-308957 (CB-030252), Towards a Coherent Strategy for Combating Biological Weapons of Mass Destruction, dated 15 April 1996.
4. Documents 3a, 3c and 3d have been deemed appropriate for release. Document 3a must have the current distribution level changed with Defense Technical Information Center (DTIC) prior to release. ECBC has no objection to the release of document 3b, however, this document requires Headquarters Department of the Army approval prior to release.

RDCB-DPC-RS

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5. The point of contact is Mr. Ronald L. Stafford, ECBC Security Specialist, (410) 436-6810 or ronald.l.stafford.civ@mail.mil.

A handwritten signature in black ink, appearing to read 'MATTHEW A. SPAULDING', written over a horizontal line.

MATTHEW A. SPAULDING
Security Manager